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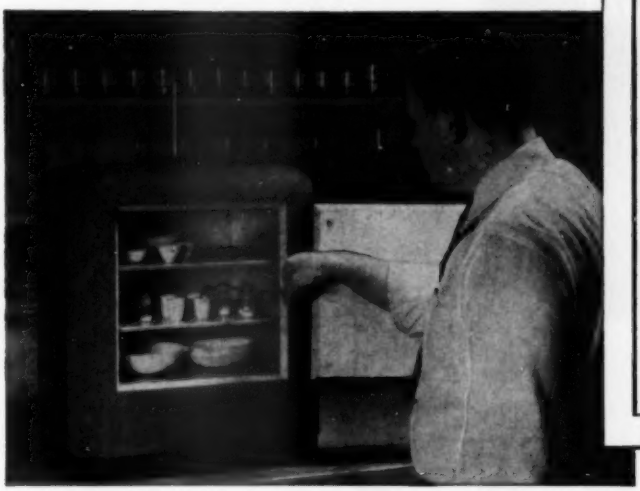

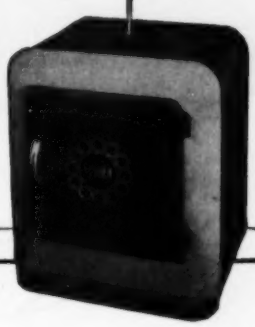

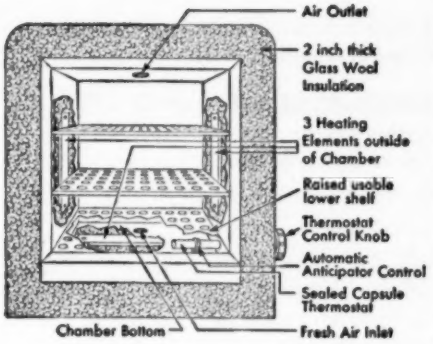

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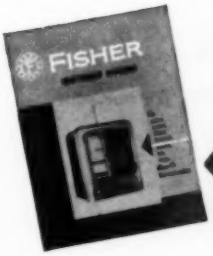

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VOLUME 57 APRIL 1954 NUMBER 4

Editorials

DR. BENNETT RESIGNS AS CHIEF EDITOR

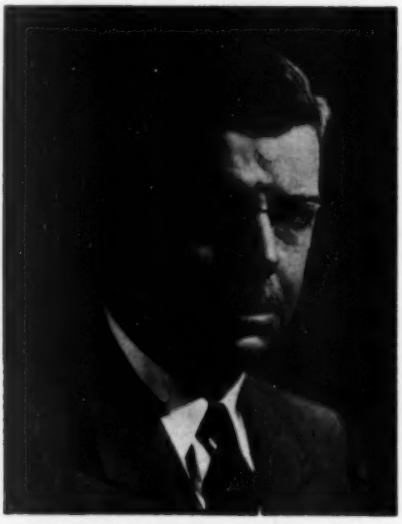
WITH SINCERE regret the resignation of Dr. Granville A. Bennett as Chief Editor of the ARCHIVES, effective March 31, 1954, is announced. Dr. Bennett, who joined the Editorial Board of the ARCHIVES in 1944 and succeeded Dr. Ludvig Hektoen as Chief Editor in 1950, has been Head of the Department of Pathology of the University of Illinois since 1944 and since his appointment on Jan. 19, 1954, Dean-Elect of the University of Illinois College of Medicine. In addition to his University appointments he is consultant to the Armed Forces Institute of Pathology and consultant in pathology to La Rabida Jackson Park Sanitarium, Chicago. The heavy responsibilities of these appointments have compelled him to give up the Chief Editorship of the ARCHIVES, although he will continue to serve as a member of the Editorial Board, and Dr. Paul R. Cannon, of the University of Chicago, has been appointed to succeed him as Chief Editor.

Dr. Cannon has had wide experience in the editorial field. He has served as Associate Editor of *The Journal of Immunology*, *The American Journal of Pathology*, and *The Journal of Laboratory and Clinical Medicine* and was a member of the editorial board of *Physiological Reviews*. He is also editor of the "American Lecture Series in Pathology" and was associated with Drs. Jolliffe and Tisdall in the editing of "Clinical Nutrition."

Dr. Cannon took over the responsibilities of Chief Editor on April 1.

DR. GRANVILLE A. BENNETT, who relinquished the post of Chief Editor of the ARCHIVES on March 31, 1954, holds the bachelor of science and doctor of medicine degrees from the State University of Iowa. Harvard University conferred an honorary master of arts degree on him in 1942. He received his specialty training as a resident physician at Peter Bent Brigham Hospital, Boston. He taught at Harvard Medical School from 1927 to 1943, starting with the rank of instructor and attaining the rank of associate professor. Prior to coming to Chicago in 1944, Dr. Bennett was Head of the Department of Pathology and Bacteriology at Tulane University. Since 1944 he has been Head of the Department of Pathology at the University of Illinois.

Dr. Bennett's research interests have been concerned chiefly with structural and functional characteristics of bones and joints and with diseases of the skeletal



DR. GRANVILLE A. BENNETT

system. He is the author of sixty-seven scientific papers and one book, "Changes in the Knee Joint at Various Ages with Particular Reference to the Nature and Development of Degenerative Joint Disease." He also has contributed sections of textbooks in pathology and surgery.

Dr. Bennett is a past president of the Chicago Pathological Society, the Illinois Society of Pathologists, the American Association of Medical Museums, and the New England Pathological Society. He is a former member of the Pathology Study Section of the National Institutes of Health. He is a fellow of the College of American Pathologists, having served as vice-president of the organization and a member of the Board of Governors. He holds membership in many other scientific groups, including the American Society for Experimental Pathology, the Society for Clinical Investigation, the Society for Experimental Biology and Medicine, and the American Society of Clinical Pathology.

DR. PAUL R. CANNON, who became Chief Editor of the A. M. A. ARCHIVES OF PATHOLOGY on April 1, 1954, has been Chairman of the Department of Pathology at the University of Chicago since 1940. He received his Ph.D. degree in Bacteriology from the University of Chicago in 1921 and his M.D. degree from Rush Medical College in 1926.

Dr. Cannon has served as President of the American Association of Pathologists and Bacteriologists, the American Association of Immunologists, the American Society for Experimental Pathology, and the Chicago Pathological Society. He is a member of the National Academy of Sciences. In 1952 he was Chairman of the Section of Pathology and Physiology of the American Medical Association and for five years was Chairman of the Pathology Study Section of the United States Public

DR. PAUL R. CANNON

Health Service. He is a former member of the Food and Nutrition Board of the National Research Council, of the Council on Pharmacy and Chemistry of the American Medical Association, and of the American Board of Pathology. In 1939-1940 he was Vice-President of the Medical Section of the American Association for the Advancement of Science. During the past ten years he also has been a consultant in various branches of the military services of the United States Government. He is a recipient of the William Wood Gerhard Medal of the Philadelphia Pathological Society and of the Ward Burdick Award-Medal of the American Society of Clinical Pathologists.

His research activities have been largely in the fields of cellular and tissue immunity and of protein and electrolyte metabolism.

OSTEOGENIC SARCOMA IN A MUSKRAT FROM AN AREA OF HIGH ENVIRONMENTAL RADIOSTRONTIUM

LOUIS A. KRUMHOLZ, Ph.D.
OAK RIDGE, TENN.
AND
LIEUTENANT COLONEL JOHN H. RUST
VETERINARY CORPS, UNITED STATES ARMY

THIS IS a report of a muskrat (Ondatra zibethica) found with an osteogenic sarcoma of the right tibiofibula. Such a tumor in itself may not be unusual, but combined with the high levels of radioactive strontium in the environment and in the bony tissue of the animal it is considered unique.

Reports of the natural occurrence of osteogenic sarcoma in wild animals are rare. Ratcliffe [1] described a tumor arising on the ulna of a baboon (Papio porcarius) with metastases to the lungs, heart, and skeletal muscle. Feldman,[2] in reviewing the literature on the occurrence of osteogenic sarcomas in domestic animals up to the year 1932, mentioned occurrences in the horse, cow, dog, sheep, cat, and chicken. Willis [3] observed several osteogenic sarcomas in dogs. Cotchin [4] reports 30 sarcomas of the bones of dogs, 27 of which were osteogenic.

Conversely, reports of osteogenic sarcomas induced by radioactive materials in laboratory animals and man are quite common. The classical work of Martland,[5] which demonstrated radium as the carcinogenic agent in radium-dial painters, was the first instance in which a radioactive substance was acknowledged to be the etiological agent for osteogenic sarcoma in man. Dunlap and co-workers [6] observed osteogenic sarcomas in 9 of 13 rats that had been fed 100 μc of radium. The tumors appeared from 253 to 426 days after administration of the radium. Heller [7] gave evidence that osteogenic sarcomas occur in rats injected intraperitonally with radiostrontium (Sr^{89}). Brues [8] observed similar sarcomas in long-term studies of mice fed on a Sr^{89} diet and noted that there was an increased incidence of tumors as the dose of radiostrontium was increased. Vaughan, Tutt, and Kitman,[9] in reviewing the biological hazards of radioactive strontium stated that bone tumors par excellence are the cause of death in chronic radiostrontium intoxication and that tumors other than those arising in or near the bone are not seen. Furthermore, although the bone tumors may be of several types, the osteogenic sarcomas predominate.

The muskrat is a common semiaquatic mammal that lives in most parts of the United States and Canada. Its food consists chiefly of stems and roots of aquatic

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From the Tennessee Valley Authority, Dr. Krumholz, Aquatic Biologist; Lieutenant Colonel Rust, Pathologist.

From The Oak Ridge National Laboratory and the University of Tennessee-Atomic Energy Commission Agricultural Research Program.

vegetation or plants growing near the water. In an emergency, muskrats will utilize almost any plant as food and at times will become carnivorous and subsist almost entirely on fish, frogs, mussels, and other aquatic animals. The breeding season in Tennessee extends from late February until midsummer. Old females have had two to three litters a season consisting of from 1 to 11 individuals. The young do not breed until the season after they are born.

The muskrat reported here was caught by plant guards of the Oak Ridge National Laboratory in the X-10 Area, Roane County, Tennessee, on March 15, 1953. At the time of capture it was noticed that the right rear leg was injured and largely useless to the animal. After having been monitored by personnel of the Health Physics Division of the Laboratory and found to be contaminated, it was turned over to the Ecological Study Section* of the Health Physics Division for radioassay. The muskrat was an adult female specimen that weighed 1,325 gm. Although captured during the height of the spring breeding season, the animal was not gravid. Another female muskrat caught nearby on March 10, 1953, was carrying three nearly full-term young.

Fig. 1.—The right hindleg showing the large mass, primarily about the dorsal portion of the tibiofibula. The leg was disarticulated at the tibiofibular-femoral joint.

Fig. 2.—The right lobe of the lungs, showing the numerous metastases. The darkened areas were hemorrhagic.

On the day following its capture the animal was killed and palpation revealed a large firm swelling or growth of the right hindleg just below the knee joint. Upon closer examination, a large tumor-mass was seen extending from the knee joint to near the distal end of the tibiofibula. Examination of the viscera revealed metastases to the lungs and both kidneys. X-ray examinations of the rest of the skeleton revealed no other abnormalities.

GROSS EXAMINATION

The gross specimens were the right hindleg disarticulated at the tibiofibular-femoral joint (Fig. 1), one kidney, and lobes from both lungs (Fig. 2). The tibiofibula at its proximal end was surrounded by a 6 by 6 cm. mass of new growth that tapered toward, but did not reach, the distal end of the bone. Grossly the tumor appeared to arise below the epiphyseal cartilage. The tumor was cut anteroventrally along the long axis of the tibiofibula and the two halves

* A cooperative project between the United States Atomic Energy Commission, the Oak Ridge National Laboratory, and the Tennessee Valley Authority.

folded outward (Fig. 3). The resulting exposed surface was light gray to pinkish-white in color and showed a complete disorganization of the muscular tissue. No new nor old hemorrhage was noted. There was a definite, but tough, irregular capsule surrounding the growth. The tumor was hard to cut but not gritty to the knife. From the incised gross specimen it did not appear that the medullary cavity of the bone had been invaded. A section of the primary tumor, including the bone shaft, was used for autoradiography (Fig. 4).

All lobes of both lungs contained deeply extending tumor masses. Frequent areas of hemorrhage and obvious necrosis were seen on the pleural and cut surfaces, and it was estimated that more than half of the lung tissue was functionally impaired. Both kidneys contained several tumor masses on the surface, and, upon being incised, the masses were found to extend deep within the parenchyma. Occasional small solitary masses, together with hemorrhage in the form of petechiae and some necrosis, were present. At least one-fourth of the kidney tissue appeared to be nonfunctional. No other metastases were found.

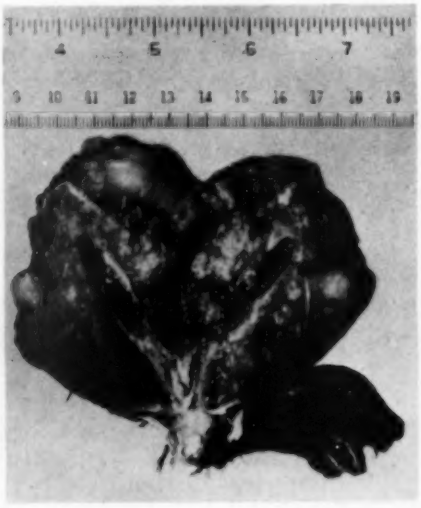

Fig. 3.—The tumor and tibiofibula reflected and exposed after being cut anteroventrally along the long axis.

MICROSCOPIC EXAMINATION

The tumor was situated for the most part outside the medullary cavity of the bone. The periosteum covered the entire growth and was seen to be reflected from the distal end of the tibiofibula by the neoplastic cells. At other points it was impossible to determine the origin of the connective tissue-like capsule. The medulla of the bone was penetrated only in a small area just below the epiphyseal plate on both sides of the metaphysis. The epiphyseal cavity was also being broached. The tumor was divided into numerous compartments by connective tissue septa, within which there were always spicules and plaques of osteoid tissue and numerous osteoblasts. However, the osteoid tissue was never calcified, nor was there any deposition of radiostrontium as shown in the autoradiogram (Fig. 5). The osteoblasts were of two types, both intimately associated with the osteoid spicules in each compartment and both laid down in a vascular bed with no particular organization. The most numer-

ous resembled osteoblasts of normal growing bone except that they occurred in large numbers about the osteoid plaques and spicules (Fig. 6). The nuclei were dark and basophilic and somewhat irregularly flattened. The cytoplasm appeared ovoid when freed from the tumor mass. The second type of osteoblasts were also closely associated with the osteoid spicules but had large, clear, vesicular nuclei. Such cells were accompanied by many bizarre nuclear forms including giant cells and double and triple nuclei. Mitotic figures were frequent.

Disoriented plates of cartilage with numerous odd cell types laid down without pattern were of frequent occurrence. In some instances, spindle-shaped cells formed plaques adjacent to sheets of osteoblasts. Such sheets of osteoblasts appeared to arise from cartilage plates.

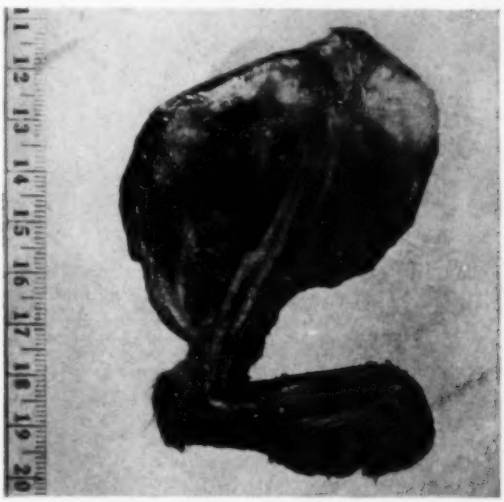

Fig. 4.—A thin slice was made of the frozen tumor and bone and exposed to Eastman N. S. film for several days. The autoradiogram obtained is shown in Figure 5.

A large peripheral nerve that was intimately involved with the periosteum enclosing the tumor was not invaded or destroyed. Two veins in the same area contained large mural tumor thrombi. Many necrotic areas were present throughout the tumor mass.

The metastatic tumor masses in the kidney contained cells similar to the neoplastic osteoblast cells of the primary tumor (Fig. 7). The nuclei were large and clear, and mitotic figures were numerous. Many small veins contained mural or occluding tumor thrombi, particularly in the capillary tufts of the glomeruli, and on one occasion a perivascular focus of tumor cells was present. In at least one instance it appeared that a glomerular embolus was the nidus for a metastatic lesion (Fig. 8). Several small lesions suggested an interstitial vascular origin. Infarction with hemorrhage and necrosis were present as a sequel to the invasion process. No osteoid or osseous tissue was observed.

Fig. 5.—Autoradiogram obtained from frozen tissue (Fig. 4). Note in particular the failure of the tumor to darken the sensitive film.

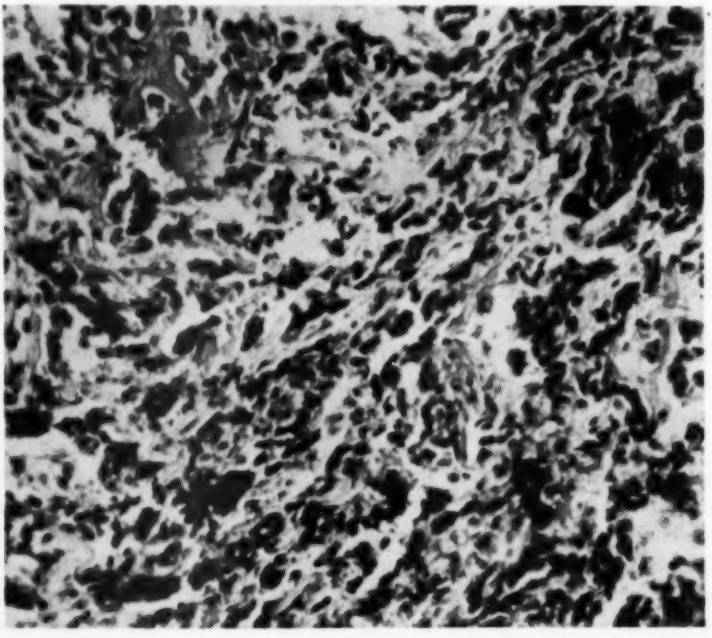

Fig. 6.—Osteoid plaques and spicules surrounded by osteoblasts. Section taken from periphery of tumor.

The lungs were massively invaded by metastases with much resultant necrosis. The cell type was similar to that found in the kidneys and vascular thrombi were also present.

The tumor was diagnosed as a sclerosing type of osteogenic sarcoma of the right tibiofibula with metastases to the lungs and kidneys.

An additional finding that was of considerable interest was the moderate degree of "severance" † between the epiphyseal plate and the spongiosa. No hemopoietic centers were found in the marrow of the affected bone, but there were such centers in long bones not involved by the neoplasm.

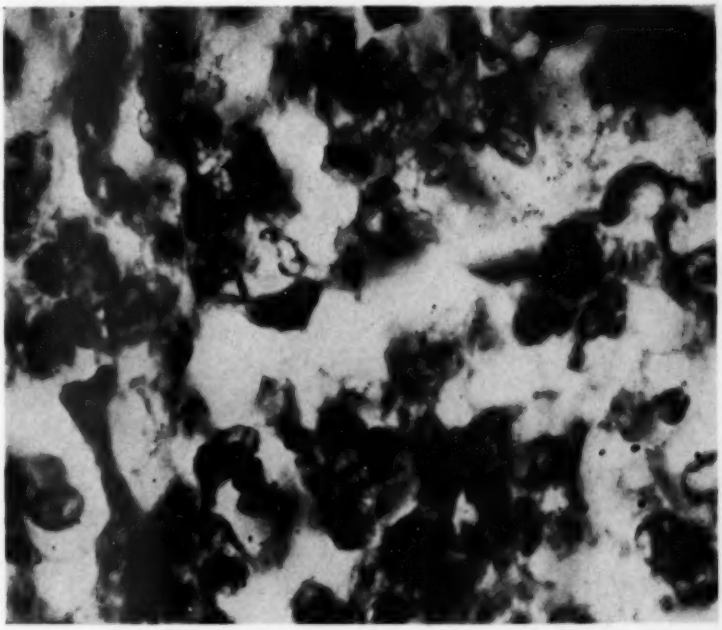

Fig. 7.—Neoplastic osteoblasts from a metastatic site in the kidney.

BIOASSAY AND RADIOCHEMICAL ANALYSIS

Samples of the various tissues were prepared for radioassay and radiochemical analysis according to the method described by Krumholz and Emmons.[10] Although most of the soft tissues of the body were sampled, none of them were found to contain amounts of radioactive materials that could have had harmful effects on the animal. However, among the hard tissues, the bone, toenails, and trachea contained unusually large amounts of radioactivity. Ordinary beta-counting techniques, using an end-window Geiger-Mueller counter, revealed that a sample of the left metatarsal bone contained 1.0 μc of radioactivity per gram of fresh tissue, and that the toenails

† Interruption of growth and continuity between the cartilage and spongy bone of the metaphysis (Heller [7]).

and trachea carried 0.35 and 0.22 μc per gram of tissue respectively. Three other samples of bone from the right femur, the left humerus, and the left metacarpals, yielded estimates of 1.2, 1.1, and 1.1 μc per gram respectively.

Absorption curves based on the data from the samples of the left metatarsal and the right femur indicated that the radioactive material present in each was largely Sr^{90} and its daughter product radioyttrium (Y^{90}). This was confirmed by radiochemical analysis. It was estimated that the entire skeleton of the muskrat contained nearly 100 μc of radioactive strontium.

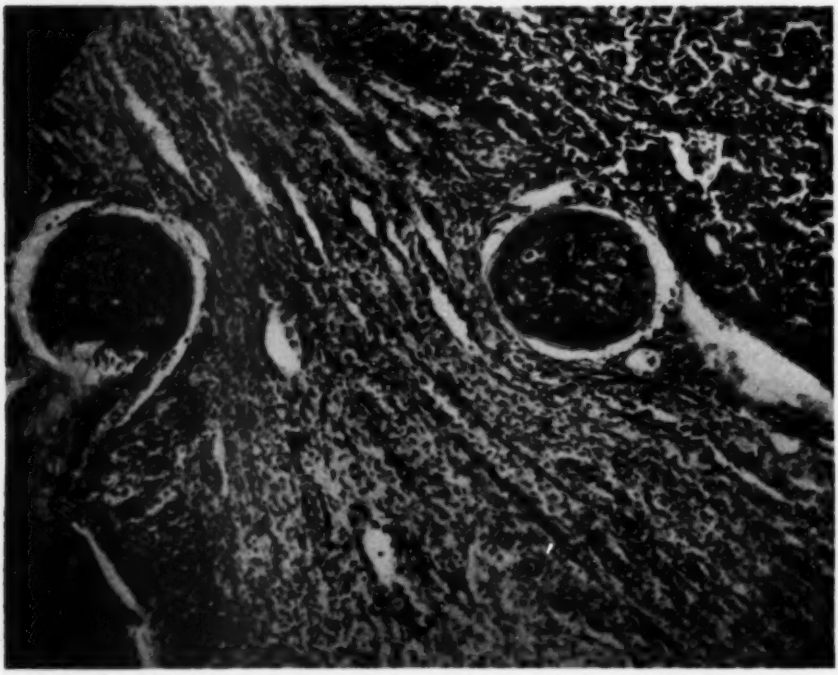

Fig. 8.—Metastases in kidney. The tumor masses in the center and at the top occupy glomerular capsules, replacing the capillary tufts. It is felt that these represent emboli.

Autoradiograms of longitudinal sections of the left femur, when compared photometrically with calibrated films, indicated that the bony tissue of the muskrat was receiving a constant dose of at least 1.5 rep per hour (40 rep per day). The duration of the exposure to the bones of the muskrat is not known, inasmuch as the source of the strontium available to the animal and the circumstances under which it was taken into the body are unknown.

Where the animal fed is unknown, but, since all nearby food sources are similar, it is believed that the following data are representative. From unpublished information gathered by personnel of the Ecological Study Section it is known that there is an abundance of food-plant material within the X-10 area of the Oak Ridge National Laboratory that contains relatively large amounts of radioelements as

indicated in the Table. These plants have been maintaining themselves in that area over a period of years and appear healthy and normal. From the data in the Table it is apparent that the different plants listed are capable of concentrating the various radioelements in amounts and percentages that are considerably different from those occurring in the water in which they were growing. Also, it is obvious that the different plants tend to show about the same percentage composition of radioactive material, although the actual amounts concentrated differed considerably from plant to plant.

Here, too, it is reasonable to assume that not more than one-third of the strontium present in any of the plant samples is in the form of Sr^{90} whereas the remaining two-thirds is Sr^{89}. Assuming that the Sr^{90} and Y^{90} are in equilibrium,

*Amounts of radioactivity in disintegrations per minute per gram of tissue (wet weight) together with the percentage composition of radiomaterials in food plants compared similar data for bones of muskrat that may have consumed such food.**

Sample	Disintegrations per Min. per Gm.	Percentage Composition of Radioelements Present			
		Ru	Sr	Rare Earths	Others
Water	16,200	40	40	14	4
Cattail leaves	17,400	1	68	21	8
Cattail leaves	15,800	1	68	22	8
Cattail leaves	7,700	3	70	26	..
Cattail leaves	8,100	2	67	24	4
Cattail rhizome	1,300	15	62	23	..
Cattail rhizome	3,300	3	63	30	4
Wild lettuce heart	21,100	2	65	26	3
Wild lettuce leaves	14,300	1	65	23	1
Curly dock stem	56,500	1	67	25	2
Curly dock stem	20,100	1	65	23	5
Curly dock heart	231,000	..	68	19	1
Curly dock heart	76,800	..	67	26	2
Muskrat metatarsus	2,310,000	..	45-50	50-55	..
Muskrat femur	2,666,000	..	45-50	50-55	..
Muskrat humerus	2,466,000	..	..	..	..
Muskrat metacarpus	2,410,000	..	..	..	..

* Samples were collected March 25, 1953. The water sampled was that in which the plants grew.

and that all of the radioactivity in the rare earths is attributable to Y^{90}, then the radioactivity emanating from the two elements should be roughly the same. The average for the radioactivity from the 12 samples listed in the Table is about 24% for the rare earths, whereas the average for strontium is about 66%. Thus, it is estimated that Sr^{90} makes up about one-third of the total and the remainder is Sr^{89}.

COMMENT

There is a high probability that the osteogenic sarcoma of the tibiofibula of the muskrat in this case report arose because of the carcinogenic action of the radiostrontium contained in the bone. The type and frequency of occurrence of this sarcoma in experimental animals plus the relatively large residual of radioactive strontium in the muskrat's bones are strong evidence in support of this belief.

It may be that the reason for the absence of radiostrontium in the neoplastic tissues in the muskrat was that the diet of the animal had been largely free of radiostrontium since the tumor started to grow, but is much more likely related to the

degree of differentiation of the tumor cells. It has been pointed out by Comar and co-workers [11] that osteoid tissue is normally quite avid for calcium and other bone-seeking elements and that there is a very close similarity between calcium and strontium in normal bone deposition. Radiostrontium consumed can be found in crystalline bone within a few hours after ingestion, and once it is incorporated in the bone there is a small but significant blood level maintained as the bone is resorbed. Therefore, if the neoplastic tissue was able to pick up radiostrontium it would have had ample opportunity to do so.

The "severance" between the epiphyseal plate and the spongiosa in the muskrat closely resembles the changes seen in the bones of cattle injected with Sr^{90}.‡ Heller [7] has observed a similar change in Sr^{89}-treated rats.

SUMMARY

The case of an osteogenic sarcoma in a muskrat (Ondatra zibethica) feeding on plants with large amounts of radiostrontium is reported. The total dose received is calculated to be at the rate of 40 rep per day. The tumors involved the right tibiofibula and was of the sclerosing type. There were metastases to the kidneys and lungs.

‡ Comar: C. L. Personal communication to the authors.

Mr. William E. Lotz and Mr. Robert Sellers, of the University of Tennessee-Atomic Energy Commission Agricultural Research Program prepared the autoradiographic, photographic, and histologic materials.

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TISSUE REACTION TO BARIUM SULFATE CONTRAST MEDIUM

Histopathologic Study

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COMPARATIVELY little has been written about human tissue reactions to barium sulfate used in roentgenographic diagnostic studies and accidentally introduced into the body cavity. Barium usually gains access to the peritoneal space during x-ray examination of the alimentary tract by way of perforated peptic ulcers clinically misdiagnosed or through penetrating peptic ulcers perforated by the diagnostic study itself. Actually, a number of reports of such gastrointestinal perforations during examination with barium meal have appeared in the literature.* By 1932, Himmelmann [5] was able to find 39 cases in the literature, and he added 5 of his own.

Interestingly enough, little attention was paid in the above-mentioned reports to reactions which barium might induce in the host. Patients dying as a result of the perforation were thought to have died from the ensuing peritonitis, and little thought was given to the possibility of whether or not the barium might worsen the immediate prognosis or subsequently give rise to troublesome granulomatous reactions should the patient survive.

In 1934, Singer [6] presented four cases of perforated peptic ulcers following roentgenographic study with barium meal. Two cases were operated upon 15 and 16 days after the perforation and peritoneal adhesions noted. No histopathologic study, however, was apparently made of visceral surfaces or adhesions.

Strasser,[7] in 1946, also gave seven illustrative examples of alimentary-tract perforations following barium studies. At the same time he referred to experimental studies in dogs carried out by Himmelmann. The latter believed that stomach contents and barium introduced into the peritoneal cavity were more deleterious than stomach contents alone. In the experimental animal, barium produced a foreign-body reaction in the peritoneum associated with severe hyperemia and exudative response.

More recently Kleinsasser and Warshaw [8] reported a case, with recovery, of a perforation of the sigmoid colon during barium enema. These authors stressed the various dangers of barium and furnished experimental studies with this element carried out in six dogs. The barium was injected into the peritoneal cavity in quantities of 20 to 150 cc. Five dogs survived and no significant adhesions resulted, with

From the Laboratory of Surgical Pathology and the Department of Surgery, Medical College of Virginia.

* References 1 to 7.

the exception of one dog. The typical pathological finding was a barium granuloma, and the authors concluded that moderate amounts of barium would not produce intestinal adhesions.

Huston, Wallach, and Cunningham [9] have studied the effect of barium sulfate experimentally in the lungs of rats. They recognized four stages in the response of the host, depending on the length of time barium was in contact with the tissues. At the end of four months, which represented the maximum length of time the experiments were conducted, the lung tissue was almost normal and no suggestion of fibrosis was present.

REPORT OF ILLUSTRATIVE CASES

CASE 1.—M. W., a 62-year-old white man, was admitted to the hospital April 20, 1951, complaining of severe nausea and vomiting. He had been a chronic alcoholic for the past

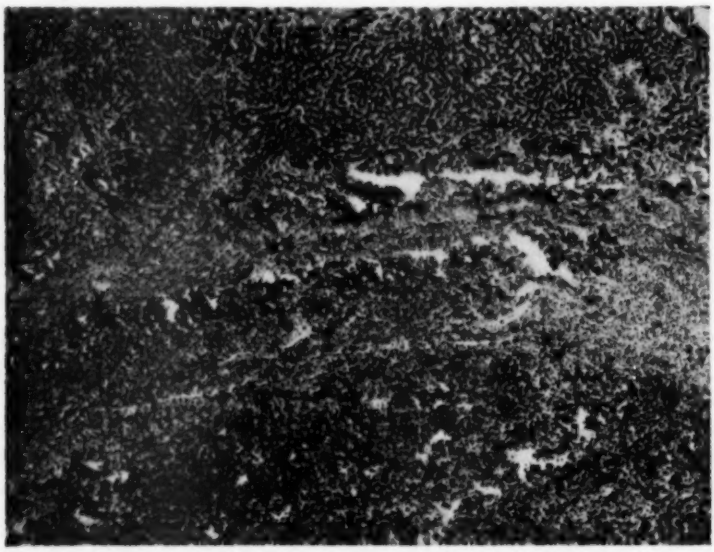

Fig. 1. (Case 1).—Marked fibrous perihepatitis with barium crystals diffusely scattered within the stroma. Note absence of involvement of liver parenchyma (× 50).

12 years. Physical examination showed tenderness in the right upper quadrant and a large mass on the anterior wall of the rectum, palpable by digital examination. A gastrointestinal series carried out on April 27, showed a duodenal ulcer with perforation into the region of the lesser peritoneal sac. This study was again repeated on May 18 and showed barium spilling into the lesser sac and extending down the posterior gutter almost to the level of the cecum. The patient was operated upon four days later, and many adhesions were found involving the right lateral gutter, right upper quadrant, and pelvis. The rectal mass, felt clinically, was inflammatory. A perforation of the anterior wall of the duodenum was found and repaired. Barium was not found in the lesser sac but rather in the region of the gall bladder and duodenum. Cardiac arrest ensued 15 minutes after the patient's return to the ward, and, despite heroic attempts at resuscitation, he died. Autopsy (A-6756) showed, in addition to perihepatitis induced by barium, coronary sclerosis, rupture of the right ventricle due to cardiac massage, and early portal cirrhosis. The liver weighed 1,500 gm. and showed a thickened capsule, particularly over the right lobe. There was a barium granuloma involving the capsule, but not extending to the parenchyma.

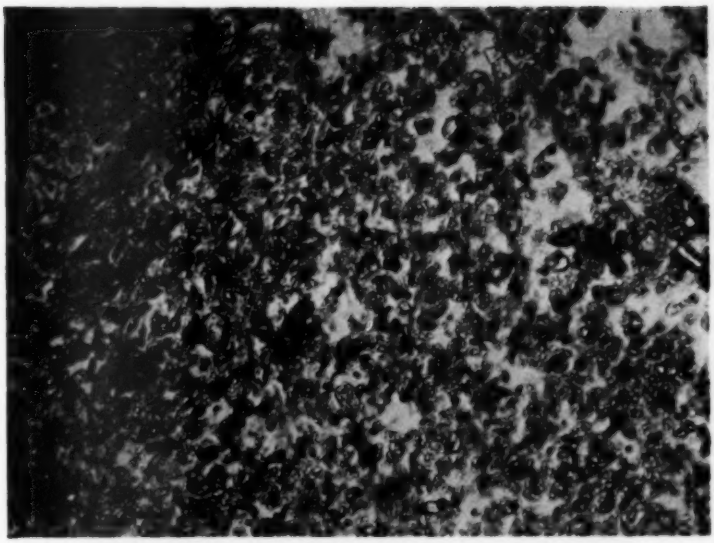

Fig. 2 (Case 1).—High-power view showing fine crystals of barium as well as coarser plates (× 200).

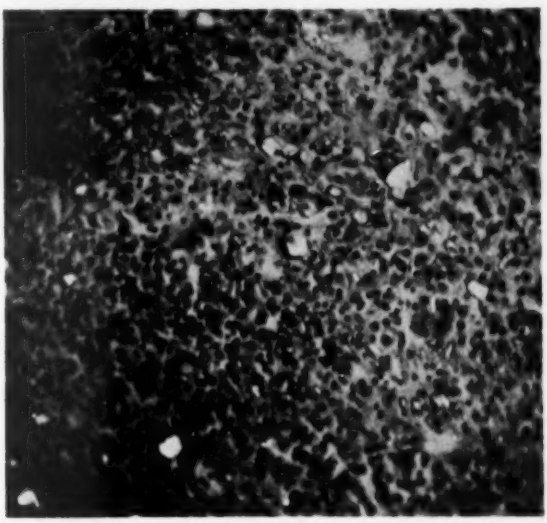

Fig. 3 (Case 2).—Barium crystals in subserosa of appendix viewed under polarized light (× 200).

CASE 2.—J. M., a 65-year-old Negro man, was admitted to the hospital May 25, 1953, with complaints of diarrhea during the past four days and right lower quadrant pain, which began four months before admission. No definite diagnosis was made, but the possibility of a right subdiaphragmatic abscess was strongly considered. On the third hospital day, 650 cc. of foul-smelling pus was withdrawn from the subdiaphragmatic space. A diagnostic barium enema June 1 showed no barium outside the intestinal tract. As there was no real improvement on tube drainage, the 11th rib was resected and open drainage instituted July 7. On July 22, a fecal odor of the material drained from the cavity led to the suspicion of a fecal fistula. This suspicion was confirmed five days later, when a barium enema demonstrated an external colic fistula arising in the ascending colon. A laparotomy was performed August 3, and a ruptured retrocecal appendix removed. Lysis of abdominal adhesions was also performed. The patient was discharged Oct. 2, 1953, free of all complaints. The appendix (S-53 5423) was 8 cm. in length and varied from 0.8 to 1.5 cm. in diameter. There was a perforation 6 cm. from the base, and

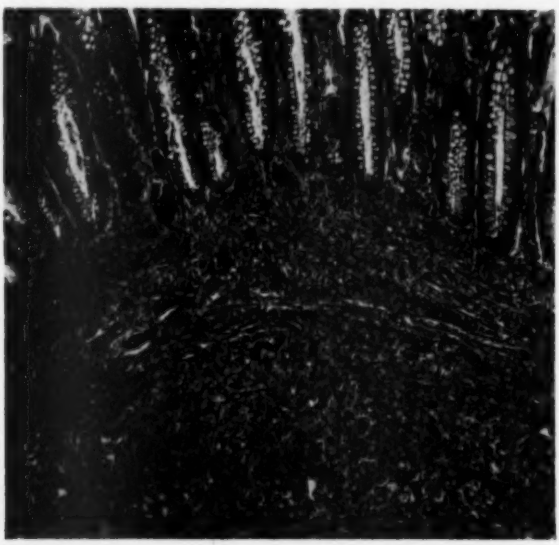

Fig. 4 (Case 3).—Submucosal nodule in rectum showing fibrous and granulomatous reaction to barium particles (× 100).

the surface was covered by fibrin and granulation tissue. Microscopically there was chronic periappendicitis and a granulomatous reaction in part due to barium.

CASE 3.†—A 53-year-old Negro man was admitted to the hospital on June 19, 1953, complaining of bright red blood in the stools of one month's duration. A complete gastrointestinal study with barium had been done May 7, 1953. Since the barium enema was unsatisfactory, this was repeated May 28. Two enemas had to be administered that day, as the first films taken were not good. The patient had no complaints during these procedures, and results of the roentgenographic study were negative. Physical examination revealed a pedunculated tumor, 1 cm. in diameter, on the posterior wall of the rectum, 6 cm. above the anal orifice. This was removed on the third hospital day (S-53 4282). Grossly this consisted of a polypoid lesion 1.2 by 1.0 by 0.3 cm. showing a rather firm, gray-white, circumscribed nodule, covered by intact rectal mucosa. The nodule was the seat of a barium granuloma.

† This case is being published in greater detail in *The Journal of the American Medical Association*.[10]

PATHOLOGIC FINDINGS

Since the pathologic changes in the three cases (liver, appendix, and rectum) were similar, they will be described together with differences noted as indicated. Amorphous pale-yellow crystals of barium sulfate were easily identified and are doubly refractile with polarized light. They were set in a stroma of proliferating fibroblasts and histiocytes. There were occasional foreign-body-type giant cells, but no tuberculid reactions were seen. Inflammatory infiltration was slight and consisted chiefly of lymphocytes and plasma cells. Case 1 showed more giant-cell response, and barium crystals could be identified in the cytoplasm of the giant cells. In Case 2, the reaction to barium was somewhat masked by the associated peritonitis due to the ruptured appendix; hence there was more severe granulation tissue and foreign-body response due to intestinal contents.

COMMENT

From the evidence at hand, it would appear that barium sulfate produces a definite but minimal fibrous tissue and inflammatory response. Actually only Case 3 showed a tissue reaction to barium uncomplicated by an associated peritonitis; hence this case represents a pure tissue response to barium. In this case the reaction led to the development of a clinical nodule thought at first to represent a neoplasm. It is quite possible that this nodule would have spontaneously disappeared had it been allowed to remain in the patient under observation.

It is difficult to ascertain from our cases the length of time needed to produce a fibrous granuloma once the tissues have been exposed to barium. Only in Case 1 can a definite time between exposure to barium and histological examination of tissue be computed, namely 25 days. In Case 2, this time interval is probably only seven days but might conceivably be as long as two months. I am not certain of the pathogenesis of the barium granuloma produced in Case 3, but the time interval may be 25 days or 6 weeks. The experimental studies of Huston and co-workers[9] in the rat showed an exudative response as early as 12 hours, but by the end of 4 months the tissues were practically normal. In the dog studies of Kleinsasser and Warshaw,[8] no information is furnished concerning the length of time the tissues were exposed to barium in each case, but it is known that at least one dog was observed as long as seven months before it was killed.

SUMMARY

Three illustrative cases of human tissue reaction to barium sulfate are presented in which the basic pathologic response was a fibrous granuloma.

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VENO-OCCLUSIVE DISEASE OF LIVER WITH NONPORTAL TYPE OF CIRRHOSIS, OCCURRING IN JAMAICA

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THE OCCURRENCE of cirrhosis of the liver in Jamaica has been reported before. McFarlane and Branday [1] described enlargement and ascites in Jamaican children, which they diagnosed as hepatitis of unknown etiology. They implied that cirrhosis of the liver might be the ultimate result. Royes,[2] in a study of 32 children, reported on the gross and histological findings in one case in which autopsy was done. His conclusions were that these cases presented a picture of portal cirrhosis very similar to that described in India and Egypt. Waterlow [3] investigated 15 cases of fatty liver disease in infants, in 4 of which autopsy was done, and he suggested that the cirrhosis which seemed to be common in children in Jamaica might be the end-result of long-standing fatty infiltration. He did not study the histological characteristics of the established cirrhosis in any detail but commented on the fact that in some of the fatty livers there was periportal proliferation of fibrous tissue, corresponding to the predominantly periportal location of the fat. Hill and co-workers * presented their findings in a condition which they termed "serous hepatosis." In 30% of approximately 75 cases studied histologically "extreme fibrosis with distortion, bile duct proliferation, and nodular hyperplasia, an appearance generally associated with cirrhosis" was seen. They suggested that a serous exudate is deposited in Disse's spaces and is subsequently invaded by fibroblastic proliferation concurrently from the portal triads and the centrilobular veins, ultimately leading to diffuse hepatic fibrosis.

The present paper deals with the morphology of a type of cirrhosis of the liver which is apparently different in one or more major aspects from those reported by the previous investigators. A striking feature in the cirrhosis presented here is its centrilobular origin and the presence of occluded branches of the hepatic veins.

MATERIALS AND METHOD

In 100 patients admitted to the University College Hospital, in hepatic as well as, to a much smaller extent, nonhepatic cases, a liver biopsy was performed with the Vim-Silverman needle. The biopsy specimens were fixed in Helly's fluid, embedded in paraffin, and 20 serial sections of each block were studied in the first instance. Hematoxylin-azophloxine, reticulin, Mallory's trichrome, periodic acid

From the Departments of Pathology and Medicine, University College of the West Indies.

* References 4 and 5.

Schiff stain, and iron stains were routinely employed. Occasionally elastic tissue, fat, and thionin stains were used. The case autopsied was studied in a similar way except that blocks of tissue from various regions of the liver were examined.

The Table shows that five patients presented occlusion of branches of the hepatic vein and centrilobular fibrosis, the condition reported here.

OBSERVATIONS

All patients were Jamaican-born and of predominantly African extraction.

CASE 1.—L. H., an 18-year-old girl, was admitted with acute ascites, accompanied by slight edema of the ankles, abdominal pain, and a firm, irregular enlarged liver, extending down to the umbilicus. There was no jaundice or pyrexia. There was no relevant past history. Her diet had always been predominantly carbohydrate, with very little protein. She was in the habit of drinking "bush teas" very frequently. Investigations were essentially negative, except that liver function tests were grossly abnormal (thymol turbidity 3.1 units, thymol flocculation 2+, gamma globulin [Kunkel] 32.1 units, choline esterase [Michel] 0.10 unit, cephalin-cholesterol flocculation 3+, plasma proteins [grams per 100 ml.] total 6.5, with albumin 2.0, and globulin 4.5).

Liver Biopsy Findings

Age Group, in Yr.	No. of Patients *		
	Examined	Showing Occlusion of Hepatic Veins and Nonportal Fibrosis	Showing Other Types of Cirrhosis
Under 1	12	..	..
1- 2	20	1	..
2- 3	6	..	..
3- 5	11	1	..
6-10	16	2	1
11-20	8	1	..
21-30	8	..	2
31-40	7	..	..
41-50	6	..	1
51 and older	6	..	..
Total	100	5	4

* There were 67 males and 33 females.

After treatment with a high protein diet, oral use of Ventriculin, and repeated paracenteses, she improved slowly over a period of six months, although when discharged she still had slight persistent ascites, a firm, nodular enlarged liver, and improved, but still abnormal, liver function tests.

Histology—Biopsy No. 1 (S 52/135): The liver parenchymal cells displayed a slight, midzonal fatty change.† Glycogen was adequately present. A very small amount of bile pigment was seen inside the cells. The nuclei of the parenchymal cells showed some irregularity in size and shape.

There was a widening of sinusoids with congestion of blood surrounding the central spaces.‡ Reticulin was condensed and partly collapsed around the central spaces and in the regions between adjacent central spaces. In these areas the radial fibers (*Radiärfasern*, von Kupffer) [10] were thickened, and the fibers encircling the sinusoids (*Gitterfasern sensu strictiori*, or *umspinnende Fasern*, Maresch) [11] were

† With this term is meant demonstration of vacuoles consistent with fatty change. Frozen sections stained with fat stains were not studied.

‡ The anatomical terms as employed by Elias (References 6 to 9) will be used.

both increased in number and thickened. Collagen increase coincided with the reticulin increase. Serous exudation in Disse's spaces (Hill and co-workers [4]) was not observed.

Several of the central spaces and sublobular veins were partly or completely occluded (Fig. 1). The material occupying the lumen appeared to be essentially the result of intimal proliferation and consisted of connective tissue cells, with an intercellular substance of fibers and membranes taking reticulin and Mallory's trichrome stains and, to a much less extent, elastic tissue stains.

Biopsy No. 2 (S 52/232): Four weeks later the occluded or partially occluded veins were seen to occupy the centers of areas with widened sinusoids, which had

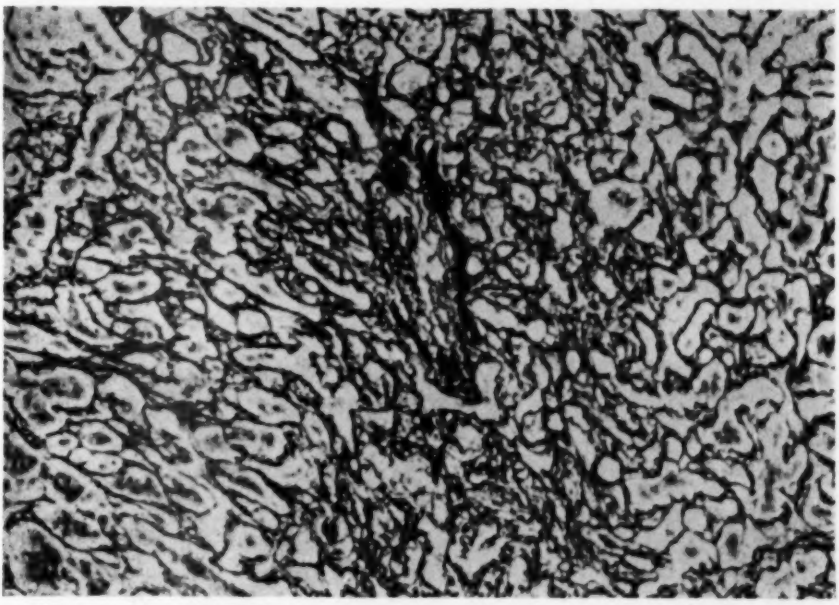

Fig. 1 (Case 1).—Occluded branch of hepatic vein surrounded by fibrosis. Reticulin stain; × 200.

the appearance of blood "lagoons"; fibrosis was coexistent in these areas. The sinusoids contained cells with atypical mitoses and occasionally karyorrhexis. Liver plates between the widened sinusoids evidently had been compressed and thinned out; occasionally they had disappeared altogether. Otherwise the appearance of the liver was as before.

Biopsy No. 3 (S 53/186): Four months after the first biopsy essentially the same picture as in the two previous biopsies was seen, but the nonportal fibrosis had progressed markedly (Fig. 2).

Case 2.—W. McD., a poorly nourished 5-year-old boy, was admitted with acute ascites, associated with a slightly irregular, firm, nontender enlarged liver (7 cm.). There was no fever or jaundice. He had always had a low protein, mainly carbohydrate diet and drank "bush teas" habitually. Investigations were essentially negative except for abnormality in some of the tests of liver function (gamma globulin 15.0 units, choline esterase 0.31 unit).

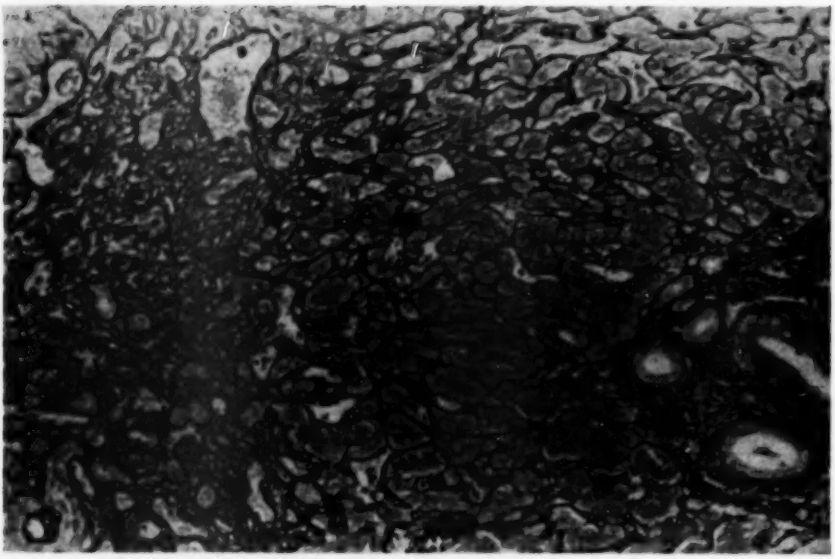

Fig. 2 (Case 1).—Nonportal fibrosis. Free zone between portal area (right) and fibrosis (left). Reticulin stain; × 200.

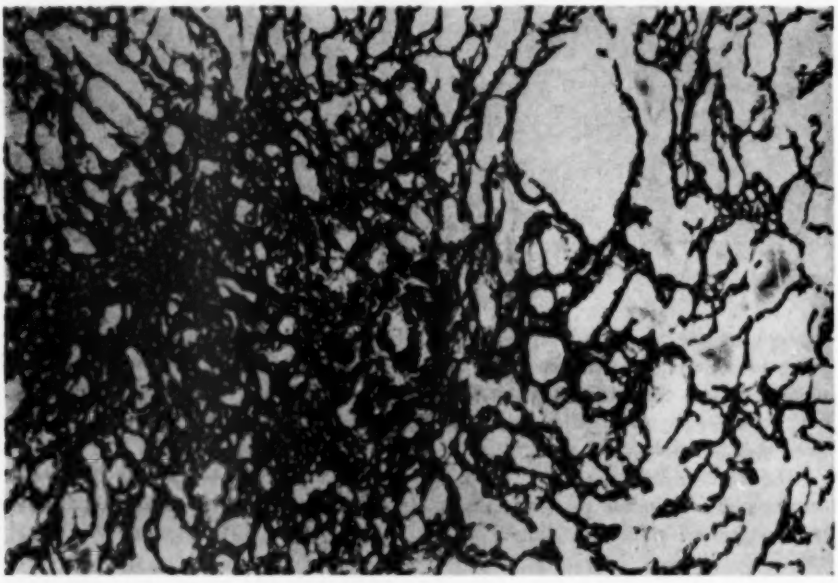

Fig. 3 (Case 2).—Occluded branch of hepatic vein surrounded by (nonportal) fibrosis. Note collateral blood space in right-upper part of picture. Reticulin stain; × 450.

Treatment with a high protein diet for a month produced a temporary improvement in the patient's clinical picture, with disappearance of ascites and diminution of the size of his liver, although the liver function tests remained essentially unchanged. This improvement was followed by a relapse, with recurrence of ascites, shortly after his return home to his previous diet.

Histology.—Biopsy No. 1 (S 52/199): As in Case 1, there was a very marked widening of sinusoids in some centrilobular areas and in the region between adjacent central spaces, coinciding with condensation of reticulin and a slight collagen increase. Again, the liver cell plates between the widened sinusoids were compressed and partly disintegrated, leaving isolated parenchymal cells with varying degrees of degeneration inside the blood "lagoons."

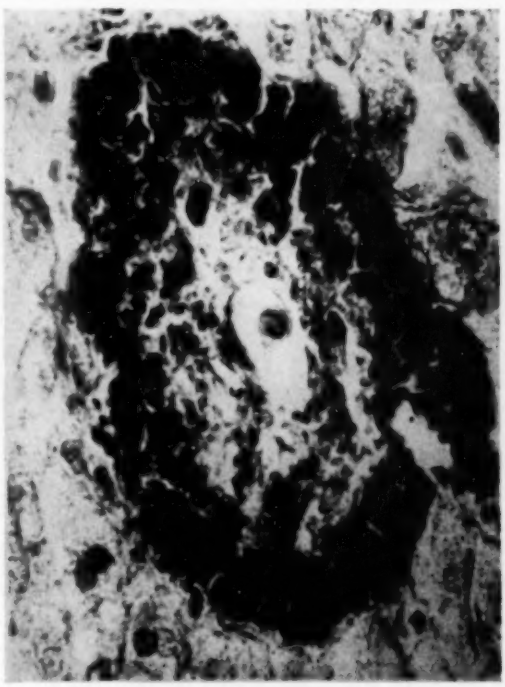

Fig. 4 (Case 2).—Branch of hepatic vein almost completely occluded, mainly due to thickening of intima. Mallory-trichrome stain; × 745.

Fat vacuoles were absent; glycogen content appeared adequate even in some of the isolated cells within the "lagoons." The nuclei of the parenchymal cells generally varied in size and shape; they displayed mitoses and evidence of amitotic division.

The reticulin condensation (similar to that in Case 1) consisted of collapse of the preexisting network, and of thickening of radial fibers with increase and thickening of encircling *Gitterfasern sensu strictiori* in the affected centrilobular areas and between central spaces, i. e., in the nonportal areas. The portal triads and periportal zones remained free.

Biopsy No. 2 (S 52/280): Four weeks after the first biopsy the cirrhosis was more advanced and was located around branches of hepatic veins, which were com-

pletely or partially occluded, mainly through intimal thickening, the composition of which varied from loose reticular tissue (Fig. 4) to denser connective tissue (Fig. 3). Inflammatory reaction was seen occasionally (Fig. 5). Fibrosis was not always concentric (Fig. 3), and not all centrilobular areas were so affected.

Widened sinusoids had developed near the obliterated vessels (Fig. 3) opposite the areas of densest fibrosis. They evidently represented a compensatory collateral circulation. A few of the parenchymal cells displayed a hyaline, eosinophilic change of cytoplasm combined with pyknosis or karyorrhexis. They were sometimes surrounded by histiocytic, lymphocytic, or eosinophilic cells. They occurred occasionally, but not exclusively, close to the fibrosed areas.

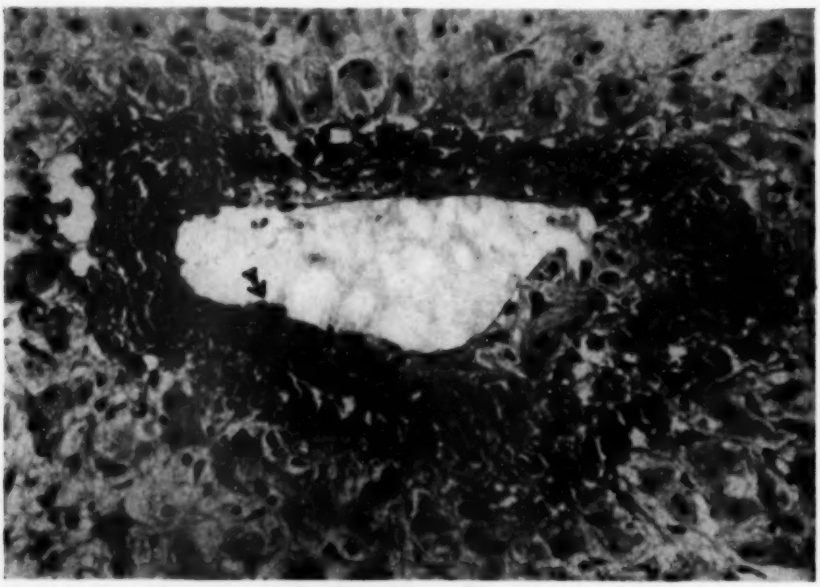

Fig. 5 (Case 2).—Obliterating endophlebitis of hepatic vein branch. Note inflammatory reaction at arrows. Mallory-trichrome stain; × 320.

The sinusoids displayed reticuloendothelial activity, but not to a large extent. Pigment was not demonstrated. Portal triads were unaffected.

Biopsy No. 3 (S 53/206); Biopsy No. 4 (S 53/306): Ten weeks and 4½ months, respectively, after the first biopsy; approximately the same picture was seen as in the previous biopsies, but there was no doubt that the cirrhosis had progressed considerably.

Case 3.—S. B., a poorly nourished 9-year-old boy, was admitted with a history of repeated hematemeses over the previous two years, associated with a hard irregular hepatomegaly (2 cm.), splenomegaly (2 cm.), and dilated veins on the abdominal wall. There was no ascites or jaundice. His past history and diet were not known. Immediate treatment was given with blood transfusions. Subsequent investigations were essentially normal save for abnormal liver function tests (bilirubin 2 gm. per 100 ml., thymol flocculation ±, gamma globulin 18.6 units, choline esterase 0.3 unit). In view of further hematemeses while in hospital, ligature of the splenic artery was carried out in an attempt to prevent further hemorrhage from esophageal varices, which had

been confirmed by esophagoscopy (Prof. G. H. C. Ovens). However, after the operation the patient's general condition deteriorated rapidly, and the patient died with bronchopneumonia and localized peritonitis on the fifth postoperative day.

Histology.—Biopsy No. 1 (six weeks after admission to hospital [S 52/286, needle biopsy]); Biopsy No. 2 (six weeks later [S 53/76, surgical biopsy during laparotomy for ligation of splenic artery]); Autopsy (nine hours after death [PM 60]): As very little morphologic difference was seen in the liver on these three separate occasions, the histology of the specimens will be discussed together.

The liver showed marked cirrhosis, finely granular in type. Collateral vessels were seen at the dome of the right liver lobe leading into the diaphragm (Fig. 6), and the ligamentum teres contained a vessel approximately 4 mm. in diameter.

On cut section a distinct lobulation was observed throughout the liver, fibrous septa dividing the parenchyma into smaller or larger areas (Fig. 7). (It will be

Fig. 6 (Case 3).—Finely granular cirrhosis. The photograph shows the dome of the right lobe with collaterals leading toward diaphragm.

emphasized below that this is a nonportal pattern.) Thickening of the wall of the medium-sized hepatic veins was seen (Fig. 7); the larger hepatic veins were relatively less affected, and the inferior vena cava was free of any gross changes.

The histopathological changes of the hepatic vein branches were seen in various stages of development. Some vessels were partially or completely occluded through a concentric padding, consisting of connective tissue showing collagen, reticulin, and elastic fibers, evidently a process of long standing (Fig. 8). Capillaries were seen surrounding, and partly within, the vessel wall. The swelling mainly concerned the intima. On the other hand, recently developed intimal thickening was seen, consisting of edematous, loose reticular tissue, showing subendothelial fibrin deposits, and proliferation of endothelial cells, with a few histiocytic and plasmocytic cells, a picture similar to that described by Teilum [12] in primary endophlebitis hepatica obliterans.

Instead of the more or less concentric pads of thickened intima, the larger veins occasionally showed irregular areas of thickening suggestive of organized thrombi.

The affected vessels, as in Cases 1 and 2, were surrounded by fibrosis, and the fibrous septa connecting these areas principally occupied nonportal zones (Hartroft [12]); they outlined islands of liver tissue showing relatively unaffected portal triads in the center (Fig. 9). However, not all portal areas were unaffected; some showed a periportal increase of reticulin and collagen, which occasionally linked up with the nonportal fibrosis. In denser fibrous areas signs of angiogenesis (Moschcowitz §) were observed.

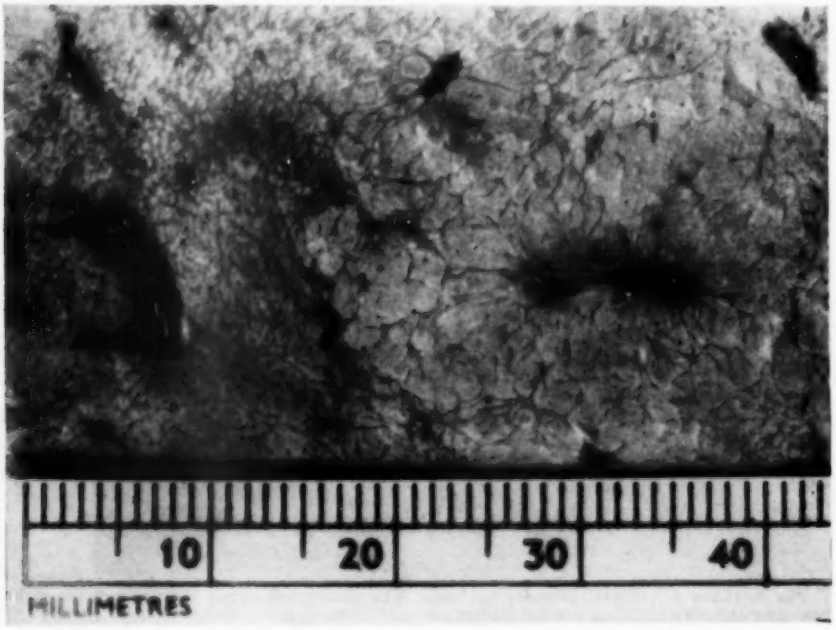

Fig. 7 (Case 3).—Cut surface of liver showing larger (right) and smaller areas (left) demarcated by fibrous septa. Note thickening of walls of hepatic veins.

Histological evidence of collateral circulation consisted of blood spaces formed through widening and coalescence of sinusoids. Often they displayed reticulin increase at one or more points on their circumference. Furthermore, portal veins were seen to be connected with vessels (by-passing or penetrating into the fibrosed areas), which were evidently formed through compensatory widening of sinusoids and of arborizations and terminal endings of portal veins. They represented portohepatic venous shunts.

Regeneration was evidenced by the formation of cell plates two or more cells in thickness (Elias [9], Popper [10]).

§ References 14 and 15.

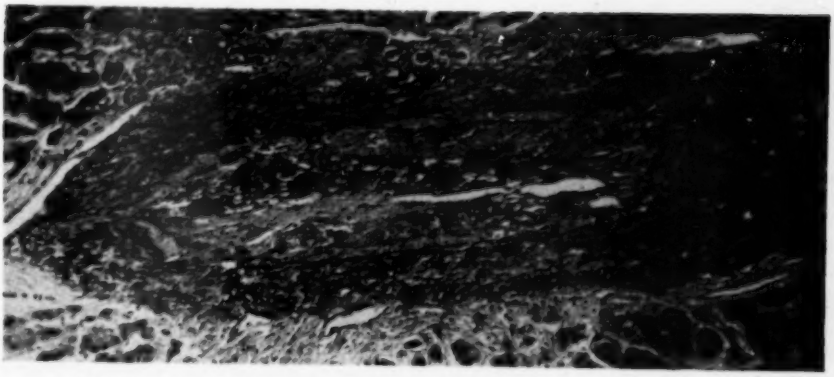

Fig. 8 (Case 3).—Branch of hepatic vein, almost completely occluded. Elastic tissue stain; × 120.

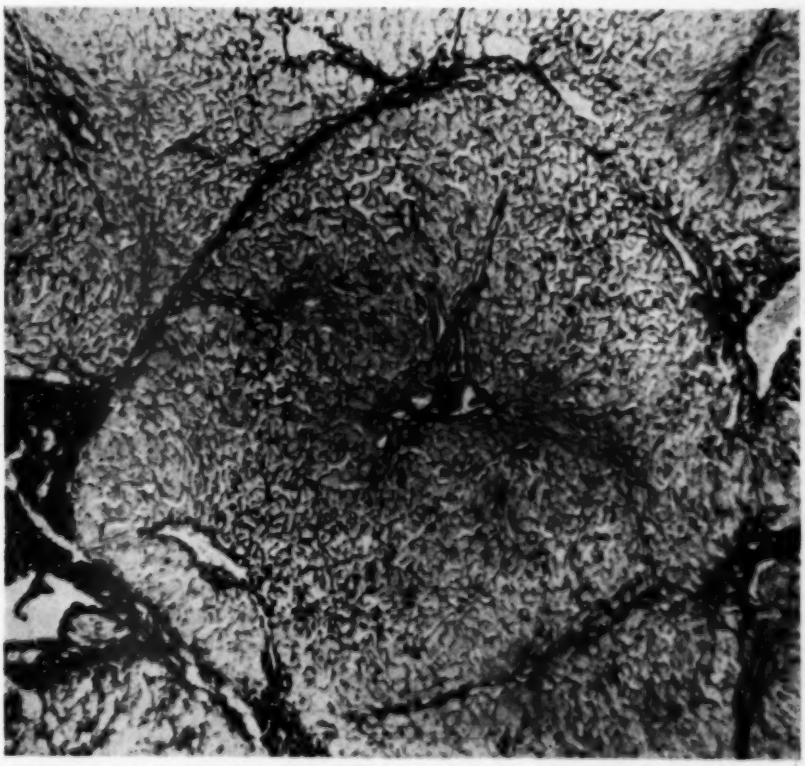

Fig. 9 (Case 3).—Fibrosis in nonportal area, outlining island of liver tissue with unaffected portal triad in the center. Reticulin stain; × 60.

The arteries in the portal triads occasionally had hyperplastic walls; there was some proliferation of bile ducts. The portal veins showed thickened walls with elastosis.

The parenchymal cells showed no fatty change. Glycogen, as shown by periodic acid Schiff stain, was variable, but apparently without predilection for particular zones of the liver lobule. The cytoplasm of some cells looked markedly empty and displayed some granularity. Liver nuclei varied in size and shape; multinucleate cells were seen, but also binucleate cells with one of the two nuclei in a pyknotic state. Occasionally there were nuclear vacuoles. Pigment was not present.

CASES 4 and 5.—Our observations include two more patients (with one and three biopsy specimens, respectively) in whom occlusion of hepatic veins was observed. One of these, Case 4, a boy aged 6 years, had an advanced cirrhosis fully conforming to a later stage of the condition described here. However, a reconstruction of the morphogenesis was impossible from biopsy material. The other, Case 5, a girl aged 1 year 11 months, who presented ascites and hepatomegaly, had occluded hepatic vein branches but as yet not more than a slight centrilobular fibrosis. This conforms to an early stage of the disease, but development into cirrhosis has to be awaited. We shall therefore at present not give more particulars of these two cases.

COMMENT

We feel justified in regarding the first three cases described here as belonging to the same morphological entity. Cases 4 and 5 appear to complement the three others, but Case 4 did not sufficiently permit a reconstruction of the pathogenesis. Cases 1 and 2 are almost identical histologically, while Case 3 shows more advanced cirrhosis. Although Cameron and Karunaratne [17] have demonstrated the reversibility of cirrhosis of the liver in experimental animals, the morphology in our cases seems to permit us to accept that a diffusely granular, cirrhotic liver can develop, initiated by occlusion of branches of the hepatic vein with subsequent centrilobular or nonportal fibrosis. The Table shows that this condition formed a substantial part of all the cases of cirrhosis seen in this group.

Obliteration of the hepatic vein radicals was an outstanding, and apparently pivotal, characteristic (Figs. 1, 3, 4, and 8). The main change was a varying amount of subendothelial, i. e., intimal, swelling, with one or more of the following characteristics: (*a*) an obliterating endophlebitis, presenting as a more or less concentric swelling of the intima, which in the earliest stage observed consisted of edematous, reticulated tissue containing fibrin and showing proliferated endothelial cells (Fig. 4) with occasionally a few inflammatory cells (Fig. 5); in later stages reticulin, collagenous, and elastic connective tissue was laid down, and (*b*) irregular swellings of the intima, suggestive of organized thrombi.‖

Slowing of the blood flow, with sinusoidal widening and compression of liver cell plates in centrilobular areas, together with collapse of the reticulin framework had resulted, subsequently followed by centrilobular and nonportal reticulin proliferation.

The fibrosis was primarily nonportal (Figs. 2, 3, 9), but in later stages the liver parenchyma was further invaded and portal areas became involved. It is hard to say whether at this stage the essentially nonportal distribution was maintained, as observed by Hartroft.[18]

‖ Duguid [16] pointed out that such thrombus organization does take place in arteries, simulating intimal thickening of arteriosclerosis, and Harrison [19] has made similar observations.

Compensatory circulation was manifested by sinusoids which had widened (Fig. 3) to form collaterals, whereas other sinusoids presumably collapsed according to the laws of Thoma.¶ Portohepatic venous shunts had developed in Case 3.

Hepatic cell injury, in the early stages caused by slowing of the circulation, may in chronic stages be effected by portohepatic shunts, which adversely influence the circulation in the center of the lobule.[16]

Collaterals to the diaphragm (Fig. 6), the ligamentum teres, and gastric and splenic vessels had developed in Case 3, apparently in a way similar to that seen in other cases of portal hypertension.

Occlusion of hepatic veins is well known and is usually referred to in literature as Chiari's, or Budd-Chiari's, syndrome.# The condition was described by Chiari * as a primary obliterating endophlebitis of hepatic veins "probably arising on a syphilitic basis," in contrast to seven cases reviewed by him which were secondary to an inflammatory process in the vicinity. Thrombosis has been a common finding in this syndrome.† Cirrhosis may secondarily, through pressure of regenerating liver nodules, compress hepatic veins and cause obliteration.‡ Hutchinson and Lévy Simpson [29] conclude from their studies that primary and/or secondary thrombosis or thrombophlebitis (such as that caused by infection, toxins, or neoplasms), mechanical factors (e. g., strain and stress of the hepatic vein), and congenital factors (atresia of hepatic veins) may give rise to this occlusion.

Except in Case 4 we may exclude thrombosis or thrombophlebitis secondary to inflammation, neoplasm, or cirrhosis of the liver. Congenital atresia or obliteration of the larger hepatic veins could be excluded in Case 3. In all five cases serological reactions for syphilis were negative, and in none were there clinical signs of congenital syphilis.

Infectious diseases and toxins have frequently been incriminated as causative factors in hepatic vein occlusion.§ Injection of extract of Ascaris suum and other substances [30] produced spasm of the hepatic veins experimentally. Maegraith and co-workers [31] held that in a wide variety of conditions, e. g., malaria and blackwater fever, a constriction of the hepatic venous tree, controlled by nervous influences, led to stagnation and anoxia in centrilobular areas of the liver. In later studies this was confirmed in cases of anaphylactic shock.||

Senecio (ragwort) poisoning may exhibit the picture of Chiari's syndrome. Selzer and Parker [34] described 12 such cases; of 5 in which autopsy was done, 3 showed thrombosis of some of the hepatic veins which was accepted as primarily due to Senecio. Fibrosis was not present, but it should be taken into consideration that the duration of the disease had been only from 5 to 10 weeks. In chronic cases of Chiari's disease fibrosis has been noted.[24] The claim by Willmot and Robertson [35] that cirrhosis of the liver developed as a result of Senecio poisoning is, we feel, not

¶ Thoma, cited by Moschcowitz (references 14 and 15).

References 20 to 24.

* References 25 and 26.

† References 20 and 23.

‡ References 27 and 28.

§ References 26 and 29.

|| References 32 and 33.

substantiated by the evidence presented. Their interesting investigations had to be terminated at an incomplete stage, and, since their patients belonged to the poorer classes, cirrhosis may well have been due to other causes.

Davidson,[36] in experimenting on rats with the Senecio alkaloid retrorsine, had observed endothelial-cell proliferation in central and sublobular veins, which he considered to be the primary effect of the alkaloid. Selzer and co-workers[37] in similar experiments, noted later that parenchymal necrosis and hemorrhage seem to occur simultaneously, and, contrary to Davidson's view, they therefore accepted the view that a primary toxic effect can be produced on both the liver cells and the vascular system. A protein-deficient diet was seen markedly to enhance the toxic effect of retrorsine. Recently Henderson and co-workers[38] demonstrated a similar toxic action for longilobine, another Senecio alkaloid.

In our patients the analogy with human and experimental Senecio poisoning is striking, but it appears from the literature that a wide variety of toxins may have this result and that it is not an exclusive feature of Senecio alkaloids. The occlusion of the smaller and medium-sized hepatic vein branches (Figs. 1, 3, 4, 5, and 8) was caused by a subendothelial, intimal thickening, which could very well be the result of toxic damage to the vascular endothelium. We feel that venous spasm, although possibly occurring in initial stages, would allow no explanation for the intimal thickening observed.

The Senecio type of poisoning in animals ¶ is not known to occur in Jamaica.# Previous investigators,* however, have repeatedly stressed the consumption in Jamaica of various types of "bush tea." These "bush teas," herbal infusions prepared from many different plants, including Senecio species, are widely used in Jamaica by the poorer classes either as a drink or medicinally, a fact emphasized by the histories of our cases.

Dietetic histories are very difficult to obtain with any accuracy in Jamaica, but it is certain that the diets of our five patients were predominantly carbohydrate, being particularly deficient in animal protein and vitamin B complex.

A comparison must be made with the infantile cirrhosis of the liver observed in India,† in which this organ is reported to be bile-stained on cut section and in which histologically ‡ the picture of a subacute toxic cirrhosis was seen, with obliteration of the terminal and some of the larger divisions of the hepatic veins. The vascular lesions were suggested to be important factors in the evolution of this type of cirrhosis.

Histologically the cirrhosis reported by us shows hepatic vein occlusion and centrilobular fibrosis strikingly similar to that described by Radhakrishna Rao §; however, we have not seen the amount of bile staining of the parenchyma and the bile cylinders described by the Indian workers, and clinically there was no jaundice. Only one of our cases was in the age group in which the Indian infantile cirrhosis is commonly seen (1 to 2 years of age).

¶ References 39 to 42.

Arnold, R. M.: Personal communication to the authors.

* References 1, 2, and 4.

† References 43 to 47.

‡ References 43 and 44.

§ References 43 and 44.

The condition has points of similarity with the one termed serous hepatosis, in which apparently some of the patients were seen to have a centrilobular fibrosis (Hill and co-workers [4] [Figs. 7 and 11]). The clinical picture in our Case 2, furthermore, was in keeping with that described by Hill and co-workers.[4] Histologically, however, serous exudation in Disse's spaces was absent in our material, and the sinusoidal lining was in close apposition to the liver cells.

It might, furthermore, be thought that hepatotoxic injury to the centrilobular areas could secondarily result in changes of the hepatic venous tree. We are, however, not aware of any such observation in experimental work with carbon tetrachloride or in centrilobular necrosis in humans.‖

Also, a consideration of the possible role of virus hepatitis is justified, since in this condition Lucké [51] demonstrated marked endophlebitis, thrombosis, and occasional complete occlusion of the afferent veins of the liver, attributed by him to liver damage and "occurring in other destructive processes involving liver parenchyma." Further, this disease is reported by some authors to lead to cirrhosis.¶ The latter, however, is variously described as being diffuse or multilobular and portal in character, but definitely unlike the fibrosis in the cases presented here.

Finally, experimental dietary cirrhosis is also of interest in this discussion. It has been extensively reported upon, and we do not propose to cite more than a few of the facts here. The nonportal distribution of this type of cirrhosis has been repeatedly stressed,# although other reports had described fibrosis in connection with the portal spaces. This nonportal origin is one of the characteristics by which experimental dietary cirrhosis in rats differs from the cirrhosis which in man is also presumed to have a dietary etiology.[62]

Our cases present a nonportal cirrhosis, but fatty change of parenchymal cells, a phenomenon preceding the experimental dietary cirrhosis, was not observed. Neither was ceroid pigment seen, although this is clearly not an essential finding in experimental dietary cirrhosis and its presence in human nutritional cirrhosis has been exceptional. We are not aware of reports of hepatic vein occlusion in experimental dietary cirrhosis.

The portal hypertension and development of varices in Case 3 are interesting in comparison with the finding of Davies [63] that, notwithstanding the amount of cirrhosis in Africa, there is no portal hypertension and that in 20 years in Uganda no deaths have been recorded from ruptured varices.

SUMMARY

Of 100 patients studied clinically and by means of needle biopsies of the liver, 5 showed nonportal fibrosis associated with obliteration of branches of the hepatic veins, a condition hitherto not reported from Jamaica. In four of these, a cirrhosis with distortion of the normal lobular architecture had developed.

The Table presents data regarding the incidence and age distribution of this condition within the group studied.

The patients presented with signs of cirrhosis, in particular hepatomegaly, ascites, hematemesis from esophageal varices in one case, but no jaundice. It is held that the obliteration of the hepatic vein branches is a very early, and possibly an

‖ References 16, 17, 24, 48, 49, and 50.

¶ References 53 to 60.

References 22, 13, 60, 61.

initial, phase of the disease. It is essentially the result of a subendothelial, i. e., intimal, thickening of the vessel wall caused by a primary obliterating endophlebitis.

A centrilobular necrosis of liver cells preceding the changes observed was not demonstrated but cannot be excluded.

The relation to various possibly allied conditions reported in the literature is discussed. The analogy to Senecio poisoning is striking, but there also seems to be close similarity with infantile cirrhosis in India. The etiology is undetermined; however, our observations have given renewed interest in the possible role played by "bush tea" in undernourished persons.

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CYTOPATHOGENICITY OF ANIMAL VIRUSES IN VITRO

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IN VIEW of the interest stimulated by the observation of the cytopathogenic effect of poliomyelitis viruses in vitro and its subsequent applications, it is pertinent at this time to review experimental work describing the action of animal viruses on cells in tissue culture. Since most of the early studies with viruses in tissue culture were not directed primarily toward the detection of cellular damage, information concerning this action of viruses on cells in these investigations is frequently fragmentary. The published data included in this compilation of recorded observations are limited, in general, to investigative work that has permitted detailed and careful examination of tissue cells infected with an animal virus in vitro. In some instances histological observations were made of tissues infected in vitro that actually represent instances of tissue survival rather than tissue cultivation. Such investigations exemplify the early recognition of virus damage to tissue cells outside the animal host which has frequently been confirmed in more recent studies of continuous-phase cellular cultures in plasma or on glass. This latter method allows for frequent examinations of cells in the living state over a greater period of time before fixation and staining. It is the aim of this review to assemble certain aspects of published investigations that, considered together, may reveal some pattern of cytopathogenic action for a group of viruses or for one virus in particular.

This review is restricted to cytopathogenic activities of viruses manifested in vitro. To avoid confusion in interpretation, cytopathogenic refers to those visible cellular changes, gross or microscopic, such as distortion and pyknosis of nucleus, increased granularity of cytoplasm, irregularity of cell shape, and cellular disintegration, which occur in a cell as a result of the growth of viruses. Viral agents that produce inclusion bodies in cells but do not induce some other recognizable disarrangement of the continuity of the tissue cells of their internal structure are not included.

In certain instances investigators have used the capacity of the tissue cells to support growth of a second virus added at a later period as a criterion of possible cellular damage produced by a virus initially present in the tissue culture. Failure of growth of the second virus was taken to indicate preexisting cell damage. This criterion may prove unreliable unless tests for interference reaction between the

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viruses used are carried out. Control tissues of equal age in tissue culture must likewise be subjected to the test conditions to see if these comparable cells will support growth of the second virus.

In evaluation of the experimental data published, several factors will require consideration: (*a*) type of tissue used in the tissue cultures and method of its examination, (*b*) host adaptation of virus strains employed in the studies, and (*c*) evidence that virus has proliferated in the cultured cells.

VIRUSES WITHOUT CYTOPATHOGENICITY

Mumps Virus.—It has been stated by Enders that an egg-adapted strain of mumps virus, which grew readily in fragments of amniotic membrane of chick embryo in the Maitland type of tissue culture, did not seem to produce any harmful effect upon the tissue cells, as there was continuing tissue growth accompanied by a fall in pH of the nutrient fluid.* The virus increased relatively slowly, usually reaching a maximum concentration in the fluid phase around the 12th day of cultivation. Virus multiplied over a period of a month or longer with a gradual decline in its rate of proliferation, as shown by egg infectivity titrations and viral hemagglutination determinations on fluid removed from the suspended cell cultures at appropriate intervals.

Influenza Virus.—Robbins and Enders [1] have shown that influenza A virus (PR8) grew well in various chick embryonic tissues in suspended cell cultures and, like mumps virus, appeared to exert no harmful effect upon the tissue cells as measured by continued growth of tissue and its capacity to support growth of a second virus. In contrast to mumps virus, influenza virus increased rapidly in the fluid phase, attaining a peak at 48 to 72 hours, as shown by both viral hemagglutination and egg infectivity titrations. Thereafter, relatively little additional virus was produced. The cause of this abrupt decrease is as yet unknown; however, Robbins and Enders [1] believe that it was probably not a result of serious injury to the host tissue, since these same cells were capable of supporting the growth of herpes simplex virus. They postulated that influenza virus rapidly exhausted some cellular factor essential for its growth but not required for the continuing existence of the host cells.

The PR8 or the Lee strain of influenza B virus in chorioallantoic or amniotic membrane fragments in balanced salt solution did not produce any significant changes in growth of fibroblasts during the observation period of 6 to 12 days.* Tissue fragments infected with virus when explanted to roller tubes exhibited fibroblast proliferation, but it is important to note that, while influenza virus is known to multiply in the ectodermal and endodermal cells of these membranes, its capacity to grow well in fibroblasts remains to be established. This question of the identity of the kind of cell in tissue cultures in which virus is multiplying is frequently perplexing when whole tissues containing a variety of cells are used for preparation of the cultures.

Yellow Fever Virus.—Fox [4] has made histological examinations of tissue from cultures of minced whole chick embryo that were infected with a chick-embryo-culture-adapted strain of yellow fever virus (17D). Sacrificing the cultures at daily intervals for five days, he found no detectable difference between cells from infected and uninfected control cultures. The supernatant fluid was removed from infected

* References 1 and 2.

cultures at the same intervals to determine multiplication of virus by titration in mice. In addition, cultures maintained for varying periods up to 40 days gave no indication that the presence of virus seriously impaired the ability of the host tissue to survive.

St. Louis Encephalitis Virus.—Harrison and Moore,[8] following the report [6] that the "Daily" strain of St. Louis encephalitis virus multiplied in suspended cell cultures of embryonic mouse tissue, demonstrated the propagation of St. Louis encephalitis virus in suspended cell cultures of chick embryo. Suspensions of infected mouse brain were used as inocula, and transplants were made at five-day intervals over a period of four months. No tissue changes attributable to the virus were observed in the cultures, although intracerebral mouse inoculations proved the presence of the virus.

Though previous investigators † obtained propagation of St. Louis encephalitis virus in suspended cell cultures, Huang [6] has shown that embryonic mouse brain tissue in tissue culture tubes exhibited unaltered growth in the presence of mouse-passage strain of St. Louis encephalitis virus, but no titrations were done to determine whether or not virus had multiplied in vitro. However, this same infected tissue did not support the growth of western equine encephalomyelitis virus when it was added later. Similar cultured tissues not infected with St. Louis encephalitis virus supported the growth of the equine virus after the lapse of a similar period of time. The action described probably represents an example of the interference phenomenon described by Duffy [9] as occurring between these two viruses.

VIRUSES WITH MODERATE CYTOPATHOGENICITY

Lymphogranuloma Venereum Virus.—Nauck [10] in 1937 and Malamos [11] in 1938 infected 5-day-old rabbit corneal epithelium in tissue cultures with lymphogranuloma venereum virus by the addition of virus-infected mouse brain fragments and observed that the membranous epithelial colonies broke up, with separation of tissue fragments into strings and islands of tissue. The first sign of infection in the cells of these fragments was the presence of a dark-blue body near the nucleus, which became granular, and also a large vacuole with numerous granules. The cultures were followed for only 48 hours after inoculation, and at that time the tissue culture fluids still produced typical symptoms of encephalitis in mice, indicating presence of virus.

Gey and Bang [12] studied the progress of an infection with lymphogranuloma venereum virus from mouse brain emulsion for more than half a year in a culture of human fibroblasts (derived from the thyroid) in roller tubes. The first cytopathological changes consisted of the formation of a few clear cytoplasmic vesicles in each colony in about seven days. When first seen the vesicles were very small. The authors felt that these small vesicles evidently represented the response of the cells to the presence of virus. Later, the vesicles became filled with granules of regular size and enlarged so much that the cytoplasm became a shell-like cover and the nucleus was pushed to one side. The enlarging vesicle caused the cell to rupture, releasing the mass of the elementary bodies. These virus particles were thus able to enter new host cells. The authors established the multiplication of virus by intracerebral inoculation of mice with fluid from the roller tubes.

† References 5 to 7.

Psittacosis Virus.—Yanamura and Meyer [13] have noted that when psittacosis virus cultivated in vitro reached a significant titer, the tissues of the culture rapidly degenerated. Morgan and Wiseman,[14] growing psittacosis virus (Strain 6BC) in chick embryo tissue in plasma-clot roller tubes, have shown that the tissue growth gradually decreased, with evidence of destruction of fibroblasts associated with virus multiplication.

Vaccinia Virus.—Rabbit corneal plasma-clot cultures incubated for 24 and 48 hours, after being inoculated with vaccinia virus [15] (Levaditi's strain of neurovaccine propagated in rabbit testicle or Noguchi's testicular virus), revealed, on histological examination, thickening of the corneal epithelium and numerous typical cytoplasmic Guarnieri bodies in the epithelial cells. In addition to the Guarnieri bodies, smaller, irregular basophilic structures, which were considered to be Paschen bodies, at times studded the cytoplasm of many cells. The end-result of the vaccinial infection was a complete dissolution of the majority of the involved cells. By testing the material for the presence of virus on the skin of normal rabbits, active vaccine virus was demonstrated in the cultures exhibiting characteristic lesions.

Enders and Florman [16] have shown that chick embryo tissue cells remain viable, as indicated by fibroblastic proliferation of explants after prolonged contact with multiplying vaccinia virus. Death of the tissue and disappearance of the virus in cultures containing vaccinia virus occurred at approximately the same time. Presence of virus was determined by titrating the fluid from the cultures on the skin of a rabbit. The percentage of tissue fragments removed from suspended cell cultures infected with virus that proved viable was definitely less, however, after the 22nd day of cultivation, as compared with tissues from control, uninoculated cultures. The authors stated that this suggests either that the virus may produce more injurious effects on cells as their vital processes became impaired, or that these differences might possibly be accounted for by variations due to random sampling.

In plasma-coated roller tube cultures,[17] using either minced whole chick embryo tissues or minced chick hearts, no apparent differences were detected by direct observation in the rate or amount of cellular growth in tubes infected with vaccinia virus, obtained in calf lymph and passed a few times on the chorioallantoic membrane of chick embryos, and that in tubes with noninfected cultures. Fragments of tissue did not lose their ability to proliferate when explanted, even though they were infected with virus for several weeks. In stained sections of tissue removed from the roller tubes, characteristic cytoplasmic inclusion bodies were demonstrated, but no evidence of changes in the cells as a result of virus growth was noted, even after contact with virus for nine weeks. Quantitative estimations of vaccinia virus were made by injecting rabbits intradermally with serial dilutions of fluid and tissue suspensions derived from cultures.

However, Robbins and Enders [1] stated in a later review of their work: "After prolonged contact, however, under suitable conditions there is some evidence that this agent is capable of injuring the cells."

Characteristic degenerative changes, comparable to cytopathogenic changes produced by poliomyelitis viruses, were described by Benedek and Kempe,[18] using the CVI strain of vaccinia virus in roller tube and Maximow double cover slip cultures of chick embryo tissue and human embryonic skin-muscle and kidney tissue embedded in a thin plasma clot. Tissue fragments were allowed to grow out a strong,

healthy mass of fibroblast-like cells before the addition of virus. After virus was added, the cells became granular and rounded and progressed to complete fragmentation and disintegration. The authors stated that specificity of virus action was observed in several experiments where specific antiserum, allowed to act on the virus, apparently had a decelerating and, in some cases, completely inhibitory effect on its cytopathogenic action.[18] The question of survival and quantitation of the virus in supernatant fluids was not investigated by the authors. The clear-cut cytopathogenic effect of vaccinia virus on chick embryo tissues observed in these experiments, as contrasted with the results of Feller, Enders, and Weller,[17] might be related to the repeated egg passage of the virus strain used by Benedek and Kempe,[18] but this, of course, would not explain its cytopathogenicity for the human tissue used.

Varicella Virus.—Utilizing plasma-clot roller tube cultures with either human embryonic skin-muscle or foreskin tissue, Weller [19] has described a cytopathogenic agent from the vesicle fluid of varicella lesions. In every instance tissue growth was well established at the time of inoculation, and focal lesions of a characteristic appearance developed from the sixth to the eighth day after inoculation. Microscopic observations of the living cultures revealed small collections of swollen, rounded, refractile cells, which contrasted sharply with the surrounding fibroblastic or epithelial outgrowth. The cells in the center of such foci gradually degenerated over the course of several days, and the destruction of cells slowly extended peripherally for days or weeks as contiguous cells became infected. Histological study of hematoxylin-and-eosin-stained cover-slip preparations from roller tubes has shown that these changes are characteristically associated with the presence of inclusion bodies. Rounded and swollen cells almost invariably possessed intranuclear eosinophilic inclusions. Attempts at in vitro neutralization of the cytopathogenic effect with convalescent serum from cases of varicella have been successful. The six agents isolated after the in vitro inoculation of varicella fluids have been propagated serially in tissue culture, as indicated by the successive development in subculture of focal areas of cellular enlargement and degeneration. These cytopathogenic changes and the development of inclusion bodies were the only evidence for the presence and multiplication of the varicella virus.

Herpes Zoster Virus.—Similar focal cytopathogenic changes were observed by Weller [19] in plasma-clot roller tube cultures of human embryonic skin-muscle and foreskin tissue inoculated with vesicle fluid from two patients with herpes zoster. Examination of stained preparations revealed that the rounded, swollen cells composing the focal lesions contained intranuclear inclusions. In attempting to distinguish this agent from herpes simplex virus, tissue suspensions prepared from these cultures were inoculated by various routes into newborn mice and produced no overt symptoms. Serial propagation of this agent has been accomplished in vitro, as evidenced by the appearance of the cytopathogenic changes in the inclusion bodies in subsequent cultures.

Herpes Simplex Virus.—Using rabbit corneal plasma-clot cultures in stationary centrifuge tubes inoculated with the HF strain of herpes simplex virus, Rivers, Haagen, and Muckenfuss [18] observed, after 24 and 48 hours of incubation, an increase in thickness of the epithelium, disappearance of intercellular bridges, swelling of cells, and appearance of many amitotic giant cells and numerous typical acidophilic

nuclear inclusions in fixed sections of cornea. Preparations similar to those fixed for histological study were emulsified and injected into the skin of rabbits, proving the presence of active virus.

A modification of the Maitland technique was used by Andrewes [20] to grow a neurotropic strain (ELI) of herpes simplex virus, maintained in rabbit brains, in Carrel flasks with minced rabbit testis. Histological examination revealed a number of changes in the cells of the tubules of the testicular tissue. The nucleolus became unrecognizable, and the whole nucleus took on a homogeneous purplish stain with hematoxylin and eosin, the nuclear membrane becoming thickened and irregularly beaded. Later the nucleus contained a granular oxyphilic body, irregular in outline, separated from the thickened nuclear membrane by a clear halo. Intratesticular, corneal, and intradermal inoculations of fluid from such cultures in rabbits revealed the presence of active virus. Furthermore, when rabbit immune serum was used instead of normal rabbit serum in the cultures, growth of the virus was prevented and no inclusions developed.

The cytologic effects of an egg-adapted herpes simplex virus on chick embryo heart and lung fibroblasts, grown under perforated cellophane in Carrel flasks without plasma in the living state and after fixation and staining, have been reported by Stulberg and Schapira.[21] Infected cultures showed focal areas of necrosis and intranuclear bodies, which were seen most frequently in cells at the periphery of a necrotic area. Viral propagation was confirmed by chorioallantoic membrane inoculation of culture fluids. A direct cytopathogenic effect of herpes virus on chick embryo lung and muscle fibroblasts in a plasma-clot roller tube and Porter flask cultures was not evident in these same studies. In one experiment, however, subcultures of cells from the infected plasma cultures did not grow as well as uninfected control plasma cultures.

In experiments reported by Scherer,[22] the addition of herpes simplex virus in infected mouse brains to the L strain of mouse fibroblasts in Porter flask cultures without plasma resulted in reduction of the cellular population. Destruction of cells was related to the presence of virus, for it had been shown that a suspension of normal mouse brain tissue did not destroy cells. Assay of virus by intracranial inoculation in mice revealed that multiplication of virus occurred when cultures were maintained at 37 C. in a medium of balanced salt solution (Hank's) with horse serum and chick embryo extract or in a medium consisting of ox serum ultrafiltrate and balanced salt solution.

Fowlpox Virus.—A strain of fowlpox virus maintained in chick embryos, when inoculated into roller tube cultures of chick fibroblasts, survived when multiplied for a period of over 100 days without causing any destruction of the fibroblasts.[23] Viral multiplication was demonstrated by inoculating the supernatant fluid on to the chorioallantoic membrane of chick embryos.

On the other hand, in roller tube cultures of chick epithelial cells, this virus destroyed the cells rapidly, while control cultures of the same cells survived indefinitely.[23] Virus titers in the supernatant fluids of epithelial cell cultures were greater than in fluids from fibroblast cultures. The possibility that fowlpox virus has a significant cytopathogenic effect on epithelial cells but not on fibroblasts was suggested, but the difference in the ease with which the virus multiplied in those two kinds of cells presents a complicating factor in the interpretation of these observations.

Newcastle Disease Virus.—When chick muscle fibroblasts grown in plasma media were exposed to Newcastle disease virus, most of the cells were destroyed within a week, but isolated strands of cells buried in the plasma clot succumbed to the virus only after the strands became accessible at one point or another.[25]

Chick chorioallantoic epithelial cells were grown on polyvinyl formal (Formvar)-coated cover slips mounted on Maximow slides for two days and then infected with the B or "vaccine" (Hitchner) strain of Newcastle disease virus.[25] After 24 or 48 hours' incubation, the tissue was fixed and examined by electron microscopy. A series of small collapsed sacs with dense areas within them were found plastered on the surface of individual epithelial cells, while neighboring cells had few or none. These sacs were identical in size, shape, and appearance with the virus found free in allantoic fluid. However, Bang found it difficult to demonstrate an over-all increase in the amount of infectious virus in the fluid from such cultures. No detailed cellular changes in the host cells were described.

In polyvinyl formal-coated roller tubes of chick chorioallantoic epithelial cells,[25] all three viruses, the virulent (CG 179), the B, and the Hitchner, or "vaccine" strain of Newcastle disease virus, multiplied readily without destroying the tissue cells. All three strains also multiplied readily in chick muscle fibroblasts but destroyed these cells, releasing virus particles.

Pseudorabies Virus.—The Hungarian strain of pseudorabies virus, maintained by rabbit passage and storage in glycerin, was cultured by Traub [26] in minced rabbit testis suspended in rabbit serum and Tyrode's solution in Florence flasks. Fixed and stained sections of tissue from cultures incubated for one day showed slight necrosis of the epithelium. No inclusions were seen. In sections from cultures incubated for two days, necrosis was more pronounced, and acidophilic intranuclear inclusions, irregular in size and sometimes in shape, appeared in the cells. Necrosis was more pronounced in cultures after three days of incubation, and inclusions were still present. The tissue was necrotic in cultures incubated for four days, and the nuclei of the cells in which inclusions usually appeared were karyorrhectic. Inclusions were no longer distinct. Mouse inoculations in every case showed that virus was present in those cultures from which pieces of tissue were removed for fixation and staining.

At the same time, Traub [26] demonstrated the cultivation of pseudorabies virus in minced chick embryo tissue suspended in Tyrode's solution with and without serum. Decimal dilutions of tissue and fluid from these cultures were injected intraperitoneally and subcutaneously into mice, demonstrating that the virus had multiplied. Czerey-Péchany, Béládi, and Iványovics [27] cultivated the virus of pseudorabies in surviving suspensions of chick embryos. The embryonal tissues were destroyed by the virus, and explants of infected embryo tissue rapidly disintegrated. Titrations of tissue culture fluids for virus, utilizing its cytopathogenic effects, yielded results in close agreement with those obtained from mouse tests.

Scherer,[28] using the L strain of mouse fibroblastic cells in Porter flasks without plasma and the Aujesky strain of pseudorabies virus, has observed a reduction in cellular population. Intracranial titrations of tissue culture fluids in mice revealed virus multiplication, which seemed to be significantly affected by rate both of cellular proliferation and of cellular destruction. Destruction of cells was related to the presence of virus, since a suspension of mouse brain tissue added to similar tissue cultures did not destroy cells.

Rabbit Virus III.—Small pieces of normal rabbit testis that had been minced in a dilution of rabbit virus III were explanted to cover slips with normal rabbit plasma and Tyrode's solution.[28] These cover slips were fixed on hollow-ground slides and incubated at 37 C. for four days. Study of the cultures directly with the microscope and of fixed sections at the end of the four days revealed that the four-day incubation was the optimal time for inclusion body formation. In cultures treated with a high dilution of virus, the radiating cellular outgrowth from the explant was disturbed in small circumscribed parts of the culture; other areas preserved the perfect alignment of radiating cells of normal control cultures. Many of the cells in the disarranged segment showed typical nuclear inclusions. In cultures with greater concentrations of virus, the cells showed more marked disturbance in growth pattern and were fewer in number, and inclusion bodies were seen everywhere. Those cultures that were heavily seeded with virus showed rapid cellular disintegration and cytolysis. Amitotic division occurred frequently in cells containing inclusions.

Coxsackie Virus.—Weller, Robbins, Stoddard, and Florentino,[29] employing the suspended cell and plasma-clot roller tube tissue culture methods, and determining virus growth by inoculation of suckling mice, observed variation in the behavior of three viruses of the Coxsackie group. The Wiederhold agent (Dalldorf's Group A), maintained in tissue culture since its isolation from human feces,[30] apparently multiplied in all the tissues tested, as evidenced by a cytopathogenic effect on human embryonic brain, intestine, and skin-muscle tissue, as well as in cultures prepared with mature human kidney and uterine tissues. Rounding, granulation, and degeneration of cells were first observed four to seven days after inoculation of the roller tube cultures. The High Point virus (Group A), maintained in mouse brain, multiplied only in suspended cell cultures of human embryonic brain tissue, as evidenced by mouse infectivity titrations. No cytopathogenic activity was observed. The DeMole virus (Group B), also maintained in mouse brain, did not appear to multiply in human embryonic skin-muscle or in mature human uterine tissue cultures, but increased significantly in cultures of human embryonic brain and intestine and of mature human kidney tissue. A definite cytopathogenic effect was noted in the cultures of brain tissue and of intestinal tissue, but not of kidney tissue, as indicated by the pH differential of infected and control cultures. Histological examination of tissue fragments revealed some degeneration of nuclei and the persistence of fewer epithelial cells in the infected tissues.

Using fragments of interscapular fat pads of newborn mice and the Conn.-5 strain (Group B) of Coxsackie virus, which is antigenically related to the DeMole virus, Stulberg, Schapira, and Eidam,[31] by a modified Porter roller flask method with plasma, have observed a direct cytopathogenic effect of the virus on fibroblasts which grew prior to viral inoculation. The progression of cytopathogenic changes was followed by microscopic observation until almost all the fibroblasts had been destroyed by the seventh day. Virus proliferation was confirmed by titrations in 2-day-old infant mice, with occurrence of a peak titer the third day and gradually decreasing titers through the seventh day after inoculation of the tissue cultures.

VIRUSES WITH MARKED CYTOPATHOGENICITY

Equine Encephalomyelitis Virus.—As an example of a virus that produces marked cytopathogenic effects in vitro, Huang [32] has shown that western equine encephalomyelitis virus completely inhibited the growth of chick embryonic skeletal muscle in

tissue culture. Explants from the tubes to plasma-coated microculture slides exhibited no outgrowth, whereas explants from uninfected cultures exhibited abundant fibroblastic outgrowths. Titrations of the supernatant fluid in mice confirmed growth of virus in the cultures.

Pieces of chick embryo tissue cultivated in plasma-clot roller tubes for six days have been exposed by Bang and Gey [88] to a chick-embryo-adapted eastern equine encephalomyelitis virus. Within 24 hours of incubation, cells of explants to polyvinyl formal-coated slides had grown out poorly, and cell destruction was evident on examination by electron microscopy. The fluid from the cultures of explanted tissues had a high titer of infectivity for 10-day chick embryos, showing that the virus had multiplied rapidly.

Using a rat fibroblast strain (14p), which originated from a subcutaneous areolar tissue explant, and another fibroblast strain (M), derived from rat muscle, Bang and Gey [34] saw no destruction of the former cells by eastern equine encephalomyelitis virus and noted marked susceptibility of the latter cells to the cytopathogenic action of the virus. The supernatant fluid from the roller tubes with and without plasma containing 14p cells did not yield virus, while that from cultures with the fibroblast strain M contained a large amount of virus, as determined by chick embryo titrations. Thus cytopathogenic effects were seen only when the virus multiplied. An intermediate type of susceptibility was characteristic for another strain of normal rat cells ("related to 14p, the normal resistant fibroblast strain"), which were derived from the articular ridges of the femur of the same rat and cultured directly on the glass wall of roller tubes. Virus multiplication and cell growth occurred simultaneously. Some cells were destroyed, but cell multiplication kept ahead of cell destruction, as indicated by continuous growth at a good rate and the necessity of replacement of nourishing supernatant fluid. Also, virus in small amounts was continuously present, as shown by titrations of supernatant fluids.

These observations indicate the importance of the precise identity of the host cells, even of the same type, in evaluating the cytopathogenic action of a given strain of a virus.

Poliomyelitis Virus.—Microscopic examination of fixed sections from human embryonic skin-muscle suspended cell cultures infected with Lansing poliomyelitis virus revealed few or no well-preserved cells.[85] "The changes observed consisted of loss of typical staining properties, nuclear pyknosis and fragmentation of cells."[85] In uninfected cultures the cells resembling fibroblasts and the nuclei of muscle cells appeared to be in good condition. The Brunhilde strain in similar cultures produced more marked cellular degeneration. Fragments explanted to plasma hanging-drop cultures from flasks in which virus multiplication was demonstrated by animal inoculation either failed to show cell proliferation or demonstrated scanty cell growth that soon showed degenerative changes. Nearly all fragments from control, uninoculated flasks developed well-defined zones of proliferation of normal-appearing cells. Also, the authors noted that acid production in the infected cultures, after a variable interval, declined more rapidly than in control cultures. Plasma-clot roller tube cultures of human embryonic skin-muscle inoculated with the Lansing or the Brunhilde strain revealed on daily examination widespread destruction of the cellular outgrowths. Specific inhibition of the cytopathogenic effect of the viruses by immune serum was demonstrated in both the flask and the roller tube culture methods.

Robbins, Enders, Weller, and Florentino [86] found that the Leon strain produced similar destructive changes in the outgrowth zone of human embryonic skin-muscle fragments in plasma-clot roller tube cultures. The poliomyelitis virus was identified by inhibition of the cytopathogenic effects with specific immune monkey serum in vitro, as well as intracerebral monkey inoculations of tissue culture fluid.

Tissue destruction produced by the three immunologic types of poliomyelitis virus was evident by gross examination of testicular tissue growing in plasma-clot roller tubes.[88] About the eighth day after implantation of monkey testicular tissue, a halo of outgrowth from these implants was seen without the aid of the microscope. This zone of growth was destroyed in the tubes subsequently infected with virus, whereas the halo of outgrowth in control tubes remained intact. Microscopically, the first evidence of virus-induced degeneration was the appearance in the periphery of the outgrowth of a zone of cells containing larger and coarser cytoplasmic granules than were found in the surrounding cells of normal appearance or in control cultures that did not contain virus. These patches of cells with the granular change spread and coalesced, giving the impression of diffuse granularity in the outgrowth zone. At the same time or shortly thereafter, various degrees of cytolysis occurred, and the normal, highly refractile appearance of the nucleus diminished and eventually was lost. The zone of fibroblastic outgrowth ceased to enlarge and usually diminished in size. In fixed and stained preparations the fibroblasts were disrupted, with loss of their normal spindle shape. A few nuclei appeared swollen and less intensely stained than in the control cultures, although the majority were shrunken and pyknotic. Virus was titered by inoculating monkey testicular tissue cultures with serial dilutions of infected tissue culture fluid and observing for the cytopathogenic effect of the virus.

Robbins, Weller, and Enders,[87] using whole mouse embryo in plasma-clot roller tube cultures inoculated with Lansing virus, did not note evidence of cytopathogenicity, and inoculations of tissue culture fluids into mice revealed no evidence of growth of Lansing virus. Also, suspended cell cultures [88] of mouse embryonic skin-muscle, brain, and intestine and of adult mouse intestine did not support the growth of Lansing virus.

VIRUSES WITH CYTOPATHOGENIC EFFECTS ON MALIGNANT TISSUE

Influenza Virus.—Pollard and Bussell,[89] using plasma-embedded tumor implants in roller tubes, have shown that influenza A virus from chick embryo chorioallantoic fluid exerted no apparent influence on a rat sarcoma or a fibrosarcoma from C_3H mice; however, a strain of influenza A virus, neurotropic in mice, produced deterioration of rat sarcoma implants. Fibrosarcoma implants from C_3H mice usually exhibited a grossly visible halo of cellular outgrowth, which equaled the size of the implant within 72 hours. Twenty-four hours after addition of these neurotropic influenza virus, the cellular outgrowths appeared granular, and some degeneration of cells was evident at 48 hours. Most of the tissues degenerated by 72 hours; however, this latter effect was not complete, since occasional healthy cells were observed. This same virus likewise caused a similar sequence of degeneration in the outgrowth from implants of an undifferentiated human parotid tumor, but the authors did not indicate whether or not any measurements were made to show that virus proliferated in any of these tumor cells.

Egypt 101 Virus.—In demonstrating the oncolytic effect of Egypt 101 virus, which is immunologically related to West Nile virus, on a human epidermoid carcinoma implanted in x-irradiated rats, Toolan and Moore [40] used an in vitro method to observe the action of the virus. Tumor was explanted from the control x-irradiated rats to cover slips that were placed in roller tube cultures. After seven days, when the tumor outgrowth was good, the tissue was exposed to mouse brain emulsion containing Egypt 101 virus. Microscopic sections 10 days after the addition of virus revealed complete lysis of the tumor cells or retraction toward the original site of outgrowth. The few fibroblasts and macrophages, representing normal rat cells in the cultures of "almost pure" tumor, appeared undamaged. The intracerebral mouse titrations of the tissue culture fluid showed that considerable multiplication of virus occurred.

St. Louis Encephalitis Virus.—This virus has an oncolytic action.[39] In plasma-clot roller tube cultures the Hubbard strain of St. Louis encephalitis virus from mouse central nervous system tissues appeared to kill explants from rat sarcoma, explants of a fibrosarcoma of C_3H mice, explants of a human undifferentiated parotid tumor, and explants of a human anaplastic adenocarcinoma within 48 hours after inoculation. The halo of cellular outgrowth showed fragmentation within 24 hours and death of all cellular elements by 48 hours. However, no titrations were carried out to indicate proliferation of the virus in these tissue cells.

In view of the fact that St. Louis encephalitis virus apparently grows readily in adult mouse tumor tissue,[39] it is interesting that Molloy [7] was not successful in attempts to grow St. Louis encephalitis virus in suspended cell cultures of adult mouse organs (brain, liver, kidney, spleen, heart, and adrenal).

Eastern Equine Encephalomyelitis Virus.—The destructive effect of a chick embryo suspension of eastern equine encephalomyelitis virus upon the rat sarcoma cells (T-333), which arose from the normal rat fibroblast strain (14p) during continuous culture when the latter was over two years old, was demonstrated by Bang and Gey [34] in roller tube cultures with and without plasma. The supernatant fluids from the sarcoma culture on the first day showed 100,000 times as much virus as was present in the normal (14p) cell cultures, which showed little evidence of virus multiplication on titration in chick embryos. By the seventh day only cell debris and dead cells were seen in the sarcoma cultures. The cultures of rat fibroblasts (14p) were at this time healthy and on direct observation showed many dividing cells.

Even in roller tubes containing both the malignant cells and the normal cells, the susceptible malignant cell colonies were rapidly destroyed, while the unsusceptible normal cell colonies continued to grow and proliferate. Titrations of fluids from these cultures showed definite multiplication of virus.[34]

Two human tumor cell strains, the D-1 Re, originally derived from a human chondromyxosarcoma in 1931 and subsequently passed through a rat, and the cell strain A Fi, in continuous cultivation since 1935, when it was explanted from a human fibrosarcoma, were destroyed either partially (A Fi) or completely (D-1 Re) by eastern equine encephalomyelitis virus. Titrations indicated virus multiplication.[34]

Poliomyelitis Virus.—Stoler and Gey,[41] using a human fibrosarcoma strain (A Fi), which has been cultivated for 15 years, have found this tissue susceptible to all three types of poliomyelitis virus in roller tube cultures without plasma. The

Lansing infection, initiated with the supernatant of a mouse brain emulsion, at the end of 60 hours reduced the cells of the outgrowth zone to cytolyzed fragments and debris. This destructive effect was observed throughout 10 in vitro passages, and the presence of the specific Lansing virus was determined by the recovery of the virus in mice and neutralization of the destructive effect by specific immune monkey serum. The destruction produced by the Leon and Brunhilde infections, initiated by tissue culture fluid from first-passage human embryo skin-muscle cultures, was similar in appearance but occurred earlier; no tissue culture passage was carried out.

All three immunological types of poliomyelitis virus have been shown to multiply in vitro in human malignant epithelial cells (Strain HeLa) derived from an epidermoid carcinoma of the cervix and maintained in continuous serial tissue culture passage.[42] The immediate source of virus for these experiments was the supernatant liquid from infected cultures of monkey testicular tissue. Propagation was observed by determining the ability of successive decimal dilutions of supernatant fluid to destroy Strain HeLa cells in vitro and also in the case of Types 1 and 3, by determining the infectivity of the supernatant culture fluids for monkeys. The propagation of each of the three types of virus was accompanied by progressive destruction of the cells, which became rounded and contracted, with pyknosis of the nucleus and granularity of the cytoplasm. Other evidence of cellular damage was the detachment of many cells from the glass substrate and total destruction of these cells in from 12 to 120 hours. These destructive effects were readily prevented by the employment of the homotypic poliomyelitis antibody but not by heterotypic antibodies.

COMMENT

In reviewing the cytopathogenic effects of viruses on cells in tissue cultures, it becomes clear not only that detailed knowledge of the virus employed, including source and host passage, is important, but also that precise information of the type of host tissue cells utilized, including their derivation and previous handling, is essential. Furthermore, unequivocal evidence for growth of virus in the cells of cultures under study, including some quantitation of this growth for comparative purposes, is necessary if any conclusions as to the effects of virus on host cells are to be reached. This is exemplified in the experiments with eastern equine encephalomyelitis virus, which causes marked destruction of rat fibroblasts of Strain M but not of rat fibroblasts of Strain 14p in tissue cultures in which virus was shown to have multiplied only in the former cells.[34] The importance of the rate of virus growth was indicated in the experiments with fowlpox virus [23] which multiplies in tissue cultures of chick fibroblasts and of chick epithelial cells, but produces obvious destruction only of the epithelial cells, in which this virus grows much more rapidly.

The recognition of the capacity of certain viruses to produce tissue cell destruction has broadened the field of in vitro culture of viruses, making possible the recognition of virus multiplication without animal titration techniques. This ready indication of virus growth in tissue culture will aid in studies of host-cell factors essential for viral proliferation, since alterations in growth conditions for host tissues can be made easily in vitro and effects on subsequent virus multiplication determined by direct observation, rather than by the more time-consuming titrations in experimental hosts.

If viruses require tissue of the human host for growth, it can be readily provided in tissue cultures; and if visible changes accompany their growth, two of the most essential requirements for their study will have been met, i. e., a suitable method for cultivation and a means for the recognition of their presence. The cytopathogenic effects of poliomyelitis and of other viruses make it possible to do neutralization tests with specific immune sera without recourse to animal or egg titrations. These developments will have special value in the study of viruses for which no known experimental host exists.

If the cytopathogenic action in tissue culture can be so standardized that a viral agent can be specifically identified, a change in pathogenicity may be recognized when the anticipated destruction of tissue is not seen. Such a variant would lend itself to further investigation in vitro, and its cytopathogenic activity for various tissues could be determined. This might lead to the selection of suitable attenuated viruses for possible use as immunizing agents.

The observation that poliomyelitis virus exhibits no exclusive neurotropic character, since it proliferates actively in extraneural tissues in tissue culture,[35] has expanded knowledge of the properties of this virus. The recognition of cytopathogenicity of other viruses for various cells in tissue cultures will enlarge the understanding of the effects of these viruses on different cells.

The exact nature of action of viruses on host cells is not clearly understood. Through in vitro demonstrations of the degenerative effect of some specific filtrable agents on certain cells, some mechanisms may be delineated. It appears that lymphogranuloma venereum virus, for example, may cause cellular destruction by physical pressure from viral proliferation within the cell walls to such a degree that the cell membranes rupture.[12] Those agents that cannot be followed microscopically in their progressive intracellular multiplication, but are seen to cause marked degeneration and destruction of host cells, may be exercising some other means of producing cell destruction. They must utilize some reactions of tissue cell metabolism or incorporate certain essential cellular constituents into their own synthesis which result in death of the host cells. This destructive action for cells in vitro demonstrates one mechanism of viral damage to host tissues but does not reveal the intricacies of the action.

The difference in the effects of a virus on normal and malignant tissues presents some interesting aspects. A most striking example of the destructive action of a virus on malignant cells is presented in the observations of the effect of eastern equine encephalomyelitis virus on rat sarcoma cells (T-333), while this virus had little if any effect on the culture of normal rat fibroblasts (14p) from which the sarcoma cells had risen.[34] In roller tube cultures of "almost pure" human epidermoid carcinoma explants from control x-irradiated rats, the few fibroblasts and macrophages, representing normal rat cells, appeared undamaged by Egypt 101 virus, while the tumor cells revealed complete lysis.[40]

In most instances reported, viruses appear to multiply more rapidly in malignant cells, and this increased rate of proliferation may account for the greater degree of cellular damage produced in general in such malignant cells. This appears to indicate some selectivity for tumor cells. The significance of such tumor selectivity will no doubt be clarified in the future and will probably contribute to the understanding of the relationship of cellular metabolism and viral growth.

On the other hand, the selectivity of viruses for malignant tissues is not universal, since Bang and Gey [24] have tested 16 cultured cell strains and find that, in general, susceptibility of cytopathogenic action of eastern equine encephalomyelitis virus is not dependent upon the malignant nature of the cell and that normal cells vary in their relative susceptibility to this virus.

The discovery of filtrable agents that possess special cytopathogenicity for tumor cells without serious destructive action on normal tissue cells may lead to the therapeutic application of viruses for effective destruction of malignant cells. However, it will be difficult to transpose results in vitro to animal hosts, where body mechanisms of resistance and special tissue susceptibility to virus action will come into play.

SUMMARY

A review of reported facts regarding virus cytopathogenicity in tissue culture is presented. The viruses have been grouped for discussion according to the effect that has been described for each virus, as follows: viruses without cytopathogenicity, viruses with moderate cytopathogenicity, viruses with marked cytopathogenicity, and viruses with cytopathogenic effect on malignant tissue.

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ELECTRON MICROSCOPY OF HUMAN SARCOMA CELLS CULTURED IN VITRO

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THE ELECTRON microscope has revealed structures in cells which cannot be seen by the optical microscope. Furthermore, when tissues are cultured in vitro, cells can be seen alone and in their entirety, and previously unsuspected details can be photographed. Differences can be seen between individual malignant and normal cells, and clues can be obtained relative to the behavior of malignant cells.

The image seen by an optical microscope can be magnified by means of various photographic methods to a magnification of × 6,000. However, the results are not comparable, because light does not resolve details beyond × 1,200. There are numerous fine processes and much cellular detail which are unresolved by light. Some of the cellular detail in malignant cells consists of numerous granules (particulate bodies or elementary bodies) which are not seen in normal cells. These granules have not been seen in the cells by light microscopy, but masses of similar granules apparently can be seen when cells are infected by various viruses.* The granules have been found associated with carcinomas.[10] They have thus been demonstrated in various animal malignant cells, but their study in human malignant cells is meager. Selby and Berger [12] found these particulate bodies in cultured neoplastic human epithelial cells. Cultured cells from human sarcomas have not been shown under the electron microscope to our knowledge. This work is presented to show electron microscope photographs of cells grown from three human sarcomas. Cells from a fourth human sarcoma were studied and photographed but are not illustrated in this report.

MATERIALS AND METHODS

Material for Tissue Culture.—The explants were taken from tissues removed from patients undergoing surgical treatment. Most of the explants were lost because of contamination during handling at operation. Animal tissues were used, as well as normal human tissues, to compare with the malignant tissues. However, it is difficult to obtain growth of normal adult tissues.

The media used for the cultures were buffered Tyrode's solution, Simm's ultrafiltrate of beef serum, Fisher's artificial medium, and dried chick embryo extract, all furnished by authorized laboratories of the Tissue Culture Association.

* References 1, 2, and 5.

Technique of Tissue Culture.—All cultures were grown in Porter flasks at 37.5 C. and were covered with a thin film of medium, as described in a previous paper.[8] The tissue was cut into 1 by 1 by 1-mm. pieces and held in place by use of a piece of No. 1 cover slip glass, which stuck to the underlying surface by surface tension, as well as thin clotting. Each flask contained 8 to 10 pieces of tissue, and as many as 50 flasks were used for culture for each tissue. The cells were cultured in a stationary position.

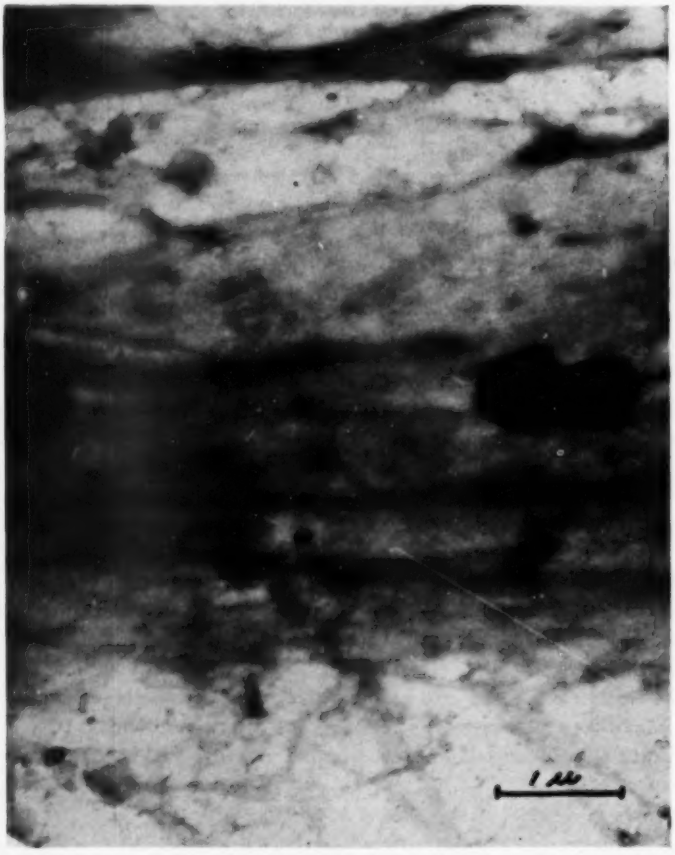

Fig. 1.—Normal cell from human testis. Sector shows fiber-like hazy cytoplasm with distinct Golgi bodies. There is a hazy cell outline, with protoplasmic extensions fusing with neighboring cells; × 17,500.

Preparation of Cells for Electron Microscope.—The method described by Porter, Claude, and Fullam [8] was used in each case. Many attempts were made to use a film which has greater advantages than a plastic film, but they were all unsuccessful. We tried silicon vapor film (quartz vapor on silver, nickel, and copper). The plastic film often breaks, or holes form, and these are often seen in the photographs. Recently we found that collodion can be used successfully and can be sterilized by

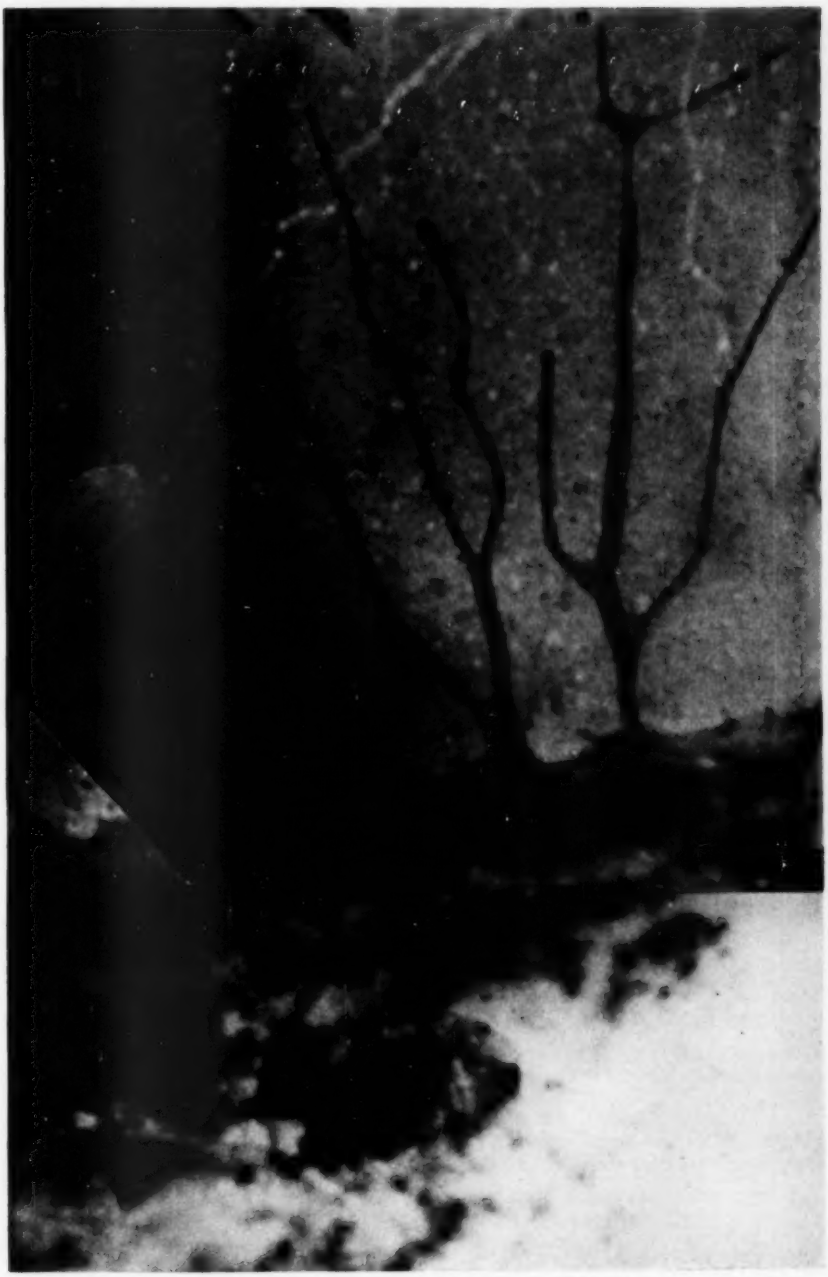

Fig. 2.—Embryonic cell from human liver. A composite electron micrograph of a cell sector. The edge shows smooth processes, and there are bodies of varying sizes throughout the cytoplasm; × 5,000.

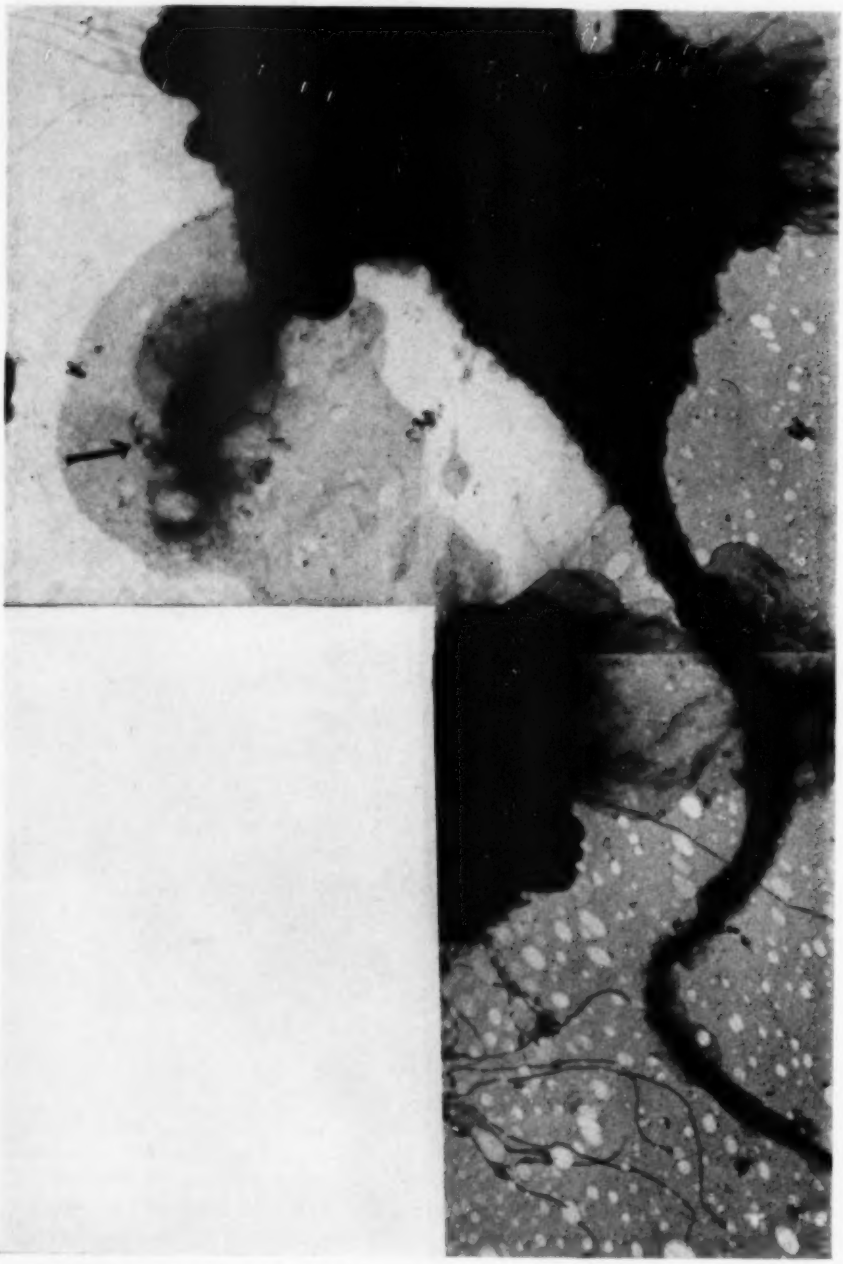

Fig. 3.—Malignant cell from mouse carcinoma. This is a composite micrograph showing one entire cell and portions of two others. One can see the long processes and veils of cytoplasm containing chains of ovoid bodies; × 1,800.

autoclaving without breaking down. The cells were grown on a polyvinyl formal (Formvar) film, and after fixation the film and cells were placed on a tantalum grid. The use of a film and a grid is necessary, since all substances, including glass and air, scatter the electrons of the electronic microscope. Thus, one must attempt to have the cells as free of all support as possible. The cells were fixed as they grew directly on the substrate film. They were not sectioned.

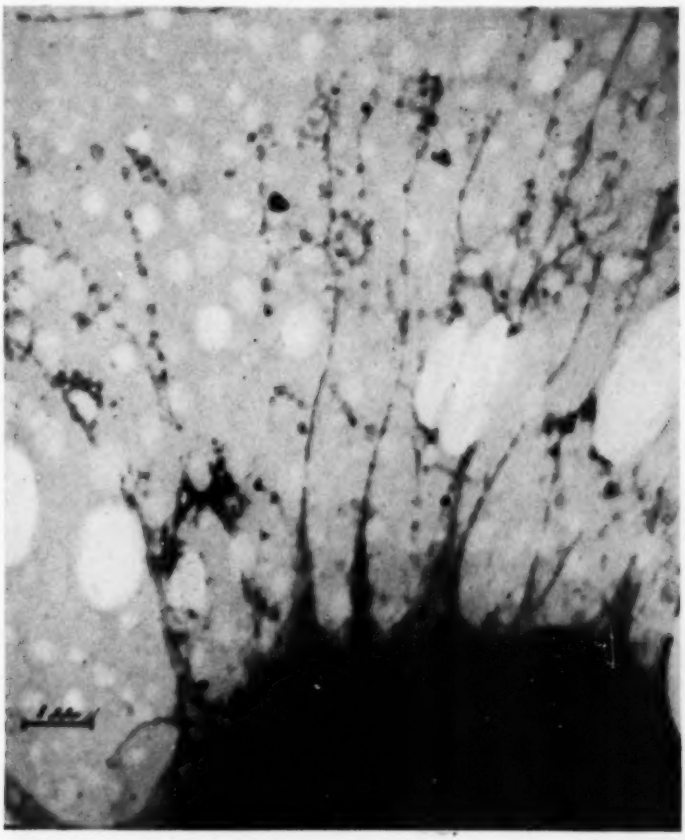

Fig. 4.—Malignant cell from human myxosarcoma. This sector shows long processes along the cell edge. One corner shows a process from another cell. The cytoplasm is densely granular and has a striped pattern; × 10,000.

The cells were fixed by exposure to osmium tetroxide for periods of 10 minutes to 24 hours. We used osmic acid vapors or covered the culture with a 2% solution of osmic acid (osmic tetroxide) in 0.85% NaCl solution in water. The cells used to illustrate this paper were washed in Tyrode's solution and fixed in 2% osmic acid in 0.85% NaCl for 20 to 30 minutes. They were washed in distilled water and dried in air. The fixation time is not critical for the appearance of the cell granules under consideration in this paper, although fixation between four to eight hours may increase the density of the granules.

All of the photographs were made with an R. C. A., type E. M. U., electron microscope, and the magnifications ranged from × 1,500 to × 4,600, with a photographic enlargement of the negative to × 5. Approximately 600 cells were photographed and studied.

OBSERVATIONS

Normal human cells grew very slowly, and often the transplants required six weeks before they showed enough growth to be photographed. The malignant cells

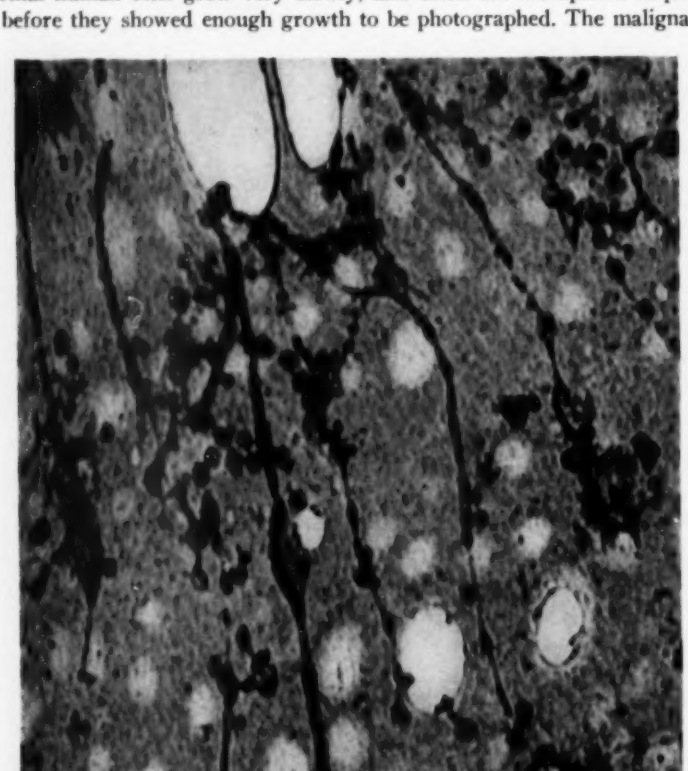

Fig. 5.—Malignant cell from human myxosarcoma. Sector showing the processes of the cell in Figure 4. They contain chains of ovoid and semicircular structures of varying density, with many dense bodies scattered throughout. A large joining process is seen from another cell; × 16,300.

spread over the entire surface of the container within a few days and grew in wild disorder. Human cells from embryos or adult inflammatory tissue grew as rapidly as malignant cells but had an orderly pattern. After two months the rate of growth was slow or had stopped, although some tissues appeared fresh grossly, even after four years in routine Porter flasks and media, and kept at 37.5 C.; however, the cells appeared hazy.

The cultures of malignant tissues usually show at least two types of cells: (1) the dominant cell type, either epithelial or sarcomatous, and (2) connective tissue or blood elements.

Many of the cells are too thick to allow electrons to pass and thus appear opaque in the electron microscope. Others spread out to a thinness which allows all of the cell except the inner structure of the nucleus to be visualized. This is true of fibroblasts and sarcomas.

Fig. 6.—Malignant cell from human liposarcoma. Sector showing processes relatively smooth and wide. There are suggestions of Golgi bodies. The ground substance has hundreds of round and ovoid bodies tending to form chains and circles; × 10,000.

RESULTS OF ELECTRON MICROSCOPY

Normal Cells.—The human cells successfully photographed were embryonic liver, adult testis, subcutaneous tissue from an infant's scalp, adult inflammatory tissue, and normal adult subcutaneous tissue.

Cells grown from human testis show a marked fibrous character, with large central bodies and with many cells having a long stringy body (Fig. 1). The processes are short and thick, and the ground substance, as well as debris, is hazy and nondescript. There are large, sharply outlined bodies surrounding the nucleus, called "Golgi bodies" by most workers.

Most of the cells grown from infant subcutaneous and adult inflammatory tissue show similar structures. The cell outline is smooth, and the nucleus is surrounded by a mass of Golgi bodies, with long mitochondria scattered throughout. The

ground substance is indistinct. An occasional cell has a thick center with some irregular processes. Mitochondria have distinct forms and are extremely long in the fibroblasts.

Human embryonic cells may have long, moderately branched processes and begin to appear like malignant cells. The ground substance is granular, and there are numerous granules which follow no definite pattern (Fig. 2). Cells from the

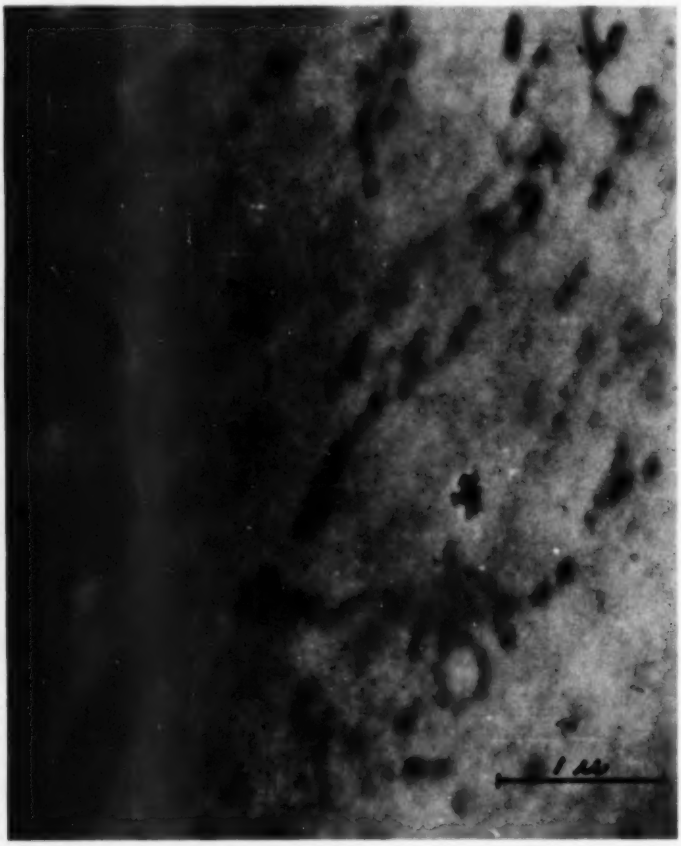

Fig. 7.—Malignant cell from human liposarcoma. The ground substance contains many ovoid bodies in chain formation. Most of the bodies are at least in pairs. The large Golgi bodies are prominent at the nucleus; × 23,000.

skin, liver, kidney, heart, spleen, and intestines were cultured from a 2-month and a 4-month human fetus. These were cultured in our early experiments, and good electron photographs were not obtained. Those that were observed in the microscope followed the same pattern.

Epithelial cells are difficult to culture and photograph in the electron microscope. They are often infected or float off the sides of the flask and are lost. They are square or cuboidal and thus are too thick to allow electrons to pass and be

photographed, except along the edges. There are numerous processes seen which may stream out in profusion. There are many cells seen with long coarse tails, from which loose thin veils seem to hang. These may be undulating membranes. These veils, when in malignant cells, contain many small ovoid bodies, and the ground substance is often granular. Numerous cells from the Barrett mouse carcinoma of the breast were grown and photographed (Fig. 3). A few human breast carcinoma

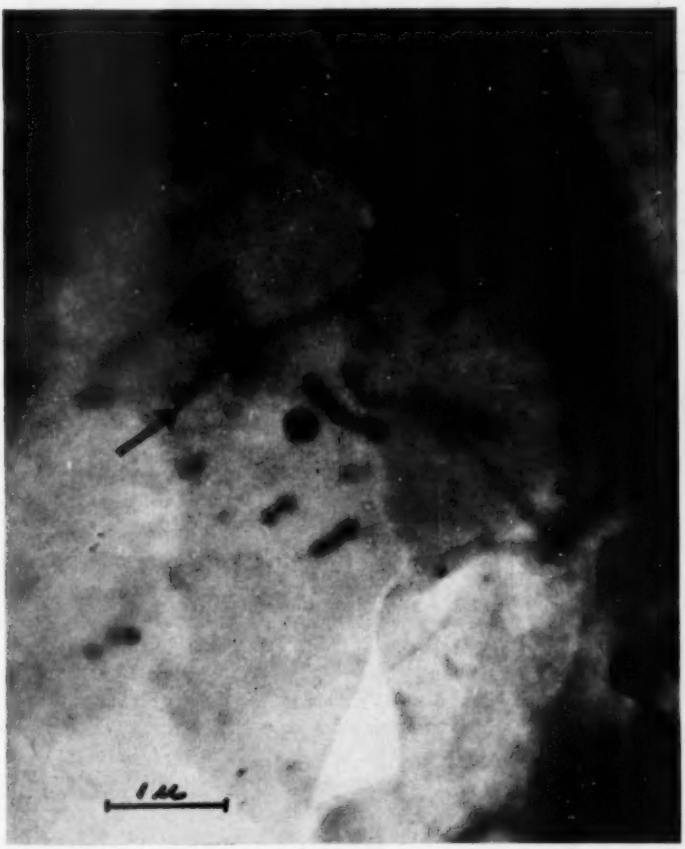

Fig. 8.—Malignant cell from human liposarcoma. This is a junction between two cells. The large dark process has been seen several times between other malignant cells. Numerous ovoid bodies are present in long chains; × 18,500.

transplants were grown, but we could not photograph them. However, their characteristics appeared the same as those of the mouse carcinoma.

Cells from human sarcomas were easy to grow, and we studied a myxosarcoma, a liposarcoma, a fibrosarcoma, and a fibromyxosarcoma. These were recurrent growths from patients 40 to 63 years of age, causing their deaths in one to four years.

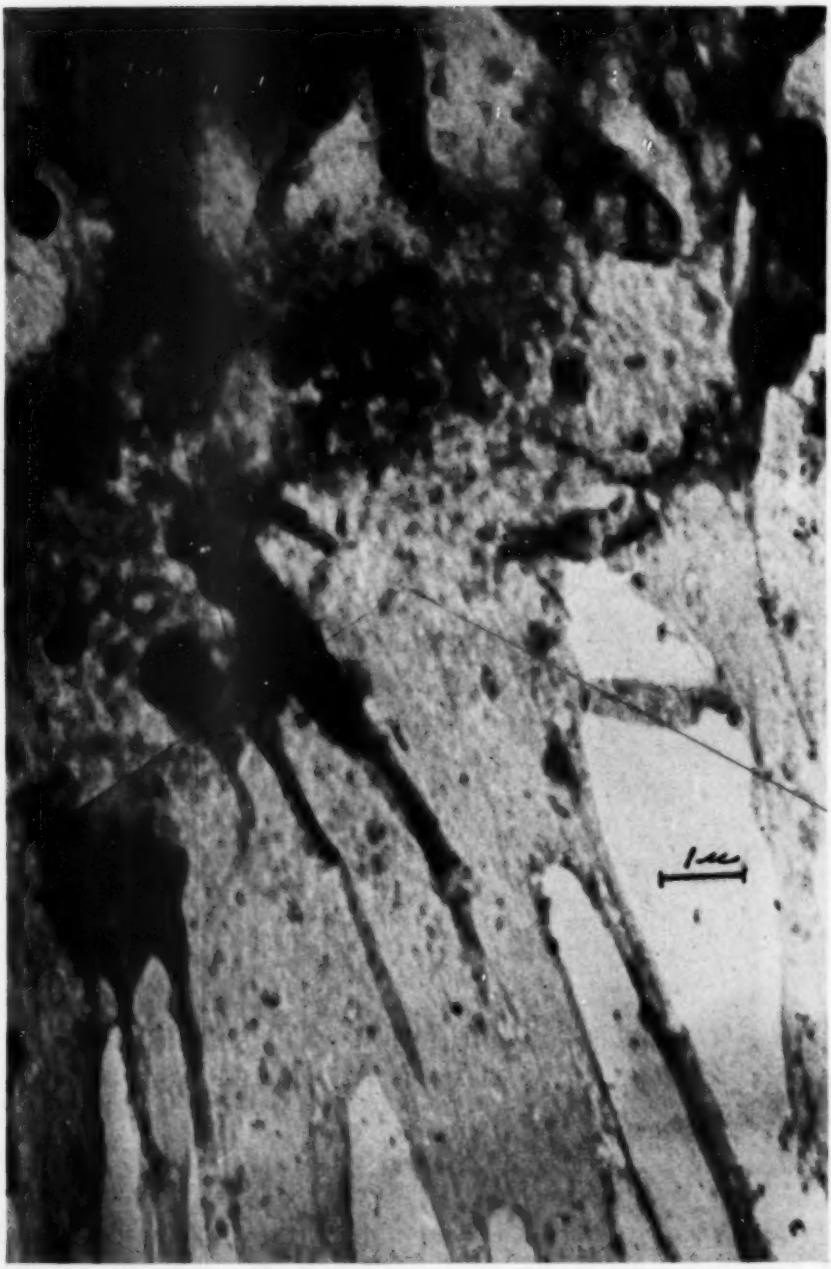

Fig. 9.—Malignant cell from human fibromyxosarcoma. Composite electron micrograph of a sector. The ground substance is filled with small thin spheroid bodies and rosettes of dense round bodies; × 13,000.

The cells from the myxosarcoma reported in this article were cultured in Porter flasks in routine media at 37.5 C. for 24 days. The liposarcoma cells were cultured for 16 days, and the fibromyxosarcoma cells were cultured for 3 days. Earlier and later growths showed no added features, and the cultures could not be carried beyond three months of growth, when they died out. Controls of the media were carried out on human fibroblasts so as to offset the possibility of virus contamination in the media. None of the normal cells showed the osmiophilic granules seen in the malignant cells, although we cultured rapidly growing cells from inflammatory tissue.

They all showed numerous long processes and granular cytoplasm. The myxosarcoma cells had extremely long processes which ended in beaded fashion, with round and semicircular densities within the beads (Figs. 4 and 5). The ground substance was heavily granular and showed a striped pattern. The cells from the liposarcoma contained numerous round and ovoid bodies averaging 100 to 150 mμ in width, and they were in chains, clumps, and rosettes. Often the chains formed circles, but the bodies were very rarely single. In many areas they appeared as single pairs (Figs. 6, 7, and 8). These bodies have not been seen in any of the several hundred nonmalignant cells observed from various sources. The fibromyxosarcoma cells (Fig. 9) contained numerous granules of varying sizes in chains and rosettes of granules. The cytoplasm was filled with a hodgepodge of granules.

COMMENT

Osmic acid is a stain as well as a fixative. It is pretty much agreed that distortion is at a minimum. Selby and Berger outlined the main characteristics that osmic acid brings out in a cell when the cell is studied in the electron microscope. One sees (1) a heavy black nucleus which is little penetrated; (2) large sharp round fat droplets and lipoproteins, often called the Golgi apparatus; (3) mitochondria in the form of filaments which are extremely long in fibroblasts; (4) endoplasmic granules which are hazy and poorly stained and are scattered throughout the cell, and (5) osmiophilic granules which are hard, round to ovoid bodies of varying densities and which are seen only in malignant cells and possibly a few in embryonic cells (Porter [9]).

The appearance of numerous extremely long processes in malignant cells suggests one reason why these cells can invade normal tissues so readily. The numerous bodies seen in our malignant cells are similar in shape, appearance, and size to many viruses which have been photographed by many observers. These can be seen as ovoid and often empty spheres. They show a varying density. That they are in pairs suggests self-proliferation. Their presence in malignant cells supports some of the virus theories regarding the etiology of malignancy.[4] The reader is referred to an excellent volume on the subject of viruses as causative agents in cancer.[6] The bodies may represent a stage in the development of a causative agent. Porter feels these should be called growth factors.[9]

The question revolves about the origin and significance of the osmiophilic granules, so far seen only in malignant cells. It is well known that they are not artefacts. Although Porter [9] feels they may be a manifestation of rapid growth, we did not see the granules in cells from human testes, inflammatory tissue which grew as rapidly as our sarcoma cells. Embryonic cells showed a few haphazard granules. Dividing cells did not show an increase in the number of granules.

It is not likely that mitochondria are the source, since these are variegated and show a tiny well-defined membrane, as well shown by Palade.[7]

Viruses are now readily photographed from many sources. Human viruses photographed by Melnick and co-workers [5] appear as round, oval osmiophilic granules which are in clumps and chains and apparently average 75 to 100 mμ in size. When these bodies are sectioned, they are found to be empty spheres.

SUMMARY

1. Connective tissue cells are so thin when grown in tissue culture that the cytoplasm can be seen clearly by electron microscopy.

2. Epithelial cells are too thick for clear definition in the electron microscope except along their edges.

3. Normal connective tissue cells have a relatively smooth outline and a hazy cytoplasm.

4. Sarcoma cells when grown in tissue culture exhibit numerous long processes.

5. Sarcoma cells often contain osmiophilic granules of the size, shape, and distribution of virus bodies, but the significance of these bodies has still to be determined.

Technical assistance was given by Bessie Levy Jacobson.

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EFFECT OF CHOLINE ON THE DEVELOPMENT AND REGRESSION OF CHOLESTEROL ATHEROSCLEROSIS IN RABBITS

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SINCE 1937 many experiments have been undertaken to determine the effect of choline on the development and regression of experimental cholesterol atherosclerosis in the rabbit.[1] Several workers have reported that the oral administration of choline inhibits, to some degree, the development of atherosclerosis in the rabbit,* or that it promotes the regression of the lesions.† On the other hand, several studies have failed to demonstrate any effect of choline on experimental atherosclerosis in the rabbit.‡ All investigators, with the exception of Morrison and Rossi [9] concede that choline is not a potent agent with respect to its alleged effects on atherosclerosis, and some of the experiments that have failed to show any effect have been criticized on the ground that an inadequate dose of choline was employed.

In these circumstances, and because of the obvious medical importance of the problem with reference to the treatment of atherosclerosis in man, further experiments were undertaken to assess the alleged effect of choline on the development and on the regression of experimental cholesterol atherosclerosis in the rabbit.

MATERIALS AND METHODS

Young adult white rabbits of both sexes weighing about 2.5 kg. were employed. They were fed 100 to 150 gm. of commercially prepared rabbit food daily. Cholesterol was added to the diet by dissolving it in ether, mixing the solution with the food, and allowing the ether to evaporate completely.[13] Choline was administered by dissolving 1 gm. of choline chloride (Merck) in 4 cc. of water and mixing this solution with the food. Choline solutions were freshly prepared daily. The choline chloride was supplied in sealed 1 oz. (28 gm.) bottles in order to minimize errors in dosage due to its hygroscopic property. The food consumption was measured daily, and the paired control animals were permitted to eat only the amount of food that was consumed by the experimental rabbits on the previous day.

Frequent chemical determinations of the serum cholesterol,[14] lipid phosphorus,[15] and total fatty acids [16] were made; the serum neutral fat [17] content was calculated from these figures. The animals were weighed at frequent intervals.

At the termination of the experiments the animals were killed, the aortas were fixed and stained in toto with Sudan IV, and the severity of the atherosclerosis was estimated visually and recorded as grade 0, 1, 2, 3, or 4. On this scale,[18] 0 indicates no grossly visible lesion;

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* References 2 to 7.

† References 8 and 9.

‡ References 1, 10 to 12.

1 indicates the presence of any lesion, no matter how minute, and an average involvement of about 10% of the intimal surface of the aorta by atherosclerotic lesions; 2 indicates about 20%, 3 about 40%, and 4 about 80% involvement of the aortic intimal surface by atherosclerosis.

Four separate experiments were undertaken. Three were experiments in which all animals were fed cholesterol and half of them also received choline. The animals were killed after two and one-half to three and one-half months of this regimen, and the severity of aortic atherosclerosis was assessed. The fourth experiment was a regression study in which all animals were fed cholesterol for three months. The cholesterol diet was stopped and one half of the rabbits were given choline chloride in their food for the subsequent five months. After this time the animals were killed and the severity of aortic atherosclerosis was determined.

RESULTS

In the first experiment 14 rabbits received 1 gm. of cholesterol daily for 93 days. Eight of the 14 animals also received 1 gm. of choline daily. The six control rabbits had aortic atherosclerosis that was graded as 1, 1, 2, 3, 3, and 4 respectively. The eight choline-treated rabbits were estimated to have atherosclerotic lesions of grades 1, 1, 2, 2, 2, 2, 3, and 4 respectively. The choline-treated rabbits suffered, on the average, involvement of about 5% less of the aortic intimal surface by atherosclerosis than the control rabbits. This difference is insignificant.

The two groups of animals had essentially the same increases in body weight and the same increase of free and total cholesterol, lipid phosphorus and neutral fat in the serum.

In the second experiment, 18 rabbits were fed 1 gm. of cholesterol daily for 75 days. Nine of the rabbits also received 2 gm. of choline daily. The grades of aortic atherosclerosis in the nine control animals at autopsy were 1, 1, 1, 2, 2, 2, 2, 3, and 3 respectively, while in the nine choline-treated rabbits they were 1, 1, 1, 2, 2, 3, 3, 3, and 3 respectively. Again, there was an average difference of involvement by atherosclerosis of 5% of the intima in the two groups. The choline-treated group was the more severely affected. The difference is negligible. There were no significant differences in the body weight or serum lipids of the control and test animals.

In the third experiment, 26 rabbits were fed 1 gm. of cholesterol three times a week for 80 to 100 days. Thirteen of the animals also received 2 gm. of choline daily. The animals were killed in pairs, the majority being killed after from 90 to 100 days of feeding. The experimental data are summarized in the Table. The 13 control rabbits had atherosclerosis of the following grades of severity: 0, 0, 1, 1, 1, 1, 2, 2, 2, 2, 2, 3, and 4. The 13 choline-treated rabbits had atherosclerosis of grades 0, 0, 0, 1, 1, 1, 1, 1, 1, 1, 2, 2, and 2 respectively. The average amount of atherosclerosis in the control rabbits represented an involvement of about 20% of the intimal surface, or Grade 2 atherosclerosis; that in the choline-treated group represented involvement of about 10% of the intimal surface or Grade 1 atherosclerosis. This is not a significant difference. Such a result might occur by chance about once in 10 trials. The weight changes in the two groups as well as the increases of cholesterol, phospholipid, and neutral fat in the serum of the control and choline-treated rabbits were essentially the same. The choline-treated rabbits gained slightly more weight than the control animals, and their serum lipids did not increase quite as much.

The fourth experiment comprised 23 rabbits that were fed 1 gm. of cholesterol daily for three months. Cholesterol feeding was stopped, and the rabbits were placed on a normal diet. After an interval of one week, the diet of 11 of the rabbits

was supplemented with 3 gm. of choline daily while the remaining 12 rabbits continued to receive normal food. Choline treatment was continued for five months after which all animals were killed.

The grades of aortic atherosclerosis observed in the 12 control animals were 2, 2, 2, 2, 3, 3, 3, 4, 4, 4, 4, and 4. The involvement of the aortas of the 11 choline-treated rabbits was graded as 1, 1, 1, 2, 2, 2, 2, 3, 3, 4, and 4. The atherosclerosis that affected the control group involved an average of about 50% of the intimal surface, while it affected the choline-treated group by involving an average of about 32% of the aortic intima. Again, this difference is not significant. The probability that the result might occur by chance is about 1 in 10. There were no important differences in body weight or in contents of lipids in the serum of the two groups of rabbits.

Average Serum Lipid Values in Cholesterol-Fed and Cholesterol-Fed Choline-Treated Rabbits: Experiment 3

	Total Cholesterol Mg. per 100 Cc.		Free Cholesterol Mg. per 100 Cc.		Lipid Phosphorus Mg. per 100 Cc.		Neutral Fat mEq./L.	
Day of Experiment	Control Animals	Choline-Treated	Control Animals	Choline-Treated	Control Animals	Choline-Treated	Control Animals	Choline-Treated
0	61.8	27.1	23.9	9.7	8.21	7.45	5.2	5.1
18	72.0	43.5	16.8	11.7	16.80	19.10	31.9	36.2
39	190.1	235.7	99.3	85.3	14.90	21.10	24.0	9.4
59	337.4	354.8	149.3	129.5	20.00	14.90	15.9	12.7
67	395.9	290.2	149.6	108.1	18.20	20.70	19.8	28.9
73	358.0	248.0	150.0	73.8	25.50	21.10	12.0	12.4
81	363.0	240.7	106.7	87.5	17.50	18.30	19.5	12.0
87	371.8	261.4	138.0	106.6	21.40	21.50	19.7	16.5
96	254.4	202.8	94.2	73.0	17.10	15.00	15.0	15.8
100	309.0	264.0	122.6	80.8	18.70	15.50	18.7	14.8

COMMENT

It was concluded from these experiments that the oral administration of choline chloride had not affected the development of experimental cholesterol atherosclerosis in the rabbit, and had not promoted the regression of the lesions. There was no apparent alteration in the serum lipids during or after cholesterol feeding that could be attributed to the ingestion of choline. The morphological character of the atherosclerotic lesions was not altered in any way by choline, either during their development or during their regression.

The results of the third experiment in which the rabbits were fed 3 gm. of cholesterol weekly and 2 gm. of choline daily, and the results of the fourth, or regression, experiment are of interest. They are similar to those obtained by Steiner § and also by Moses and Longabaugh.[7] At the time his experiments were published Steiner considered that choline delayed the development of atherosclerosis and promoted its regression. Moses and Longabaugh suggested that the supposed inhibitory effect that they observed might be due, in some part, to the fact that their choline-treated rabbits did not gain as much weight as the control rabbits.

In our paired feeding experiments, the weight gains in the experimental and in the control animals were essentially the same.

The present experiments have previously been interpreted by us ‖ to indicate that choline in appropriate dosage has a slight inhibitory effect on the development

§ References 2 and 5.

‖ References 19 and 20.

of atherosclerosis induced by small doses of cholesterol but that it does not affect the regression of the lesions. However, in view of subsequent experiments in which the method of visual grading of the severity of atherosclerotic lesions has been assessed, the conclusion that choline possesses an inhibitory effect can no longer be entertained.

It has been found that the visual method of grading the severity of atherosclerosis is crude and that it is a justifiable method of assessing data only if the numbers of animals considered are large enough to avoid sampling errors and to permit adequate statistical treatment or if the difference in the severity of atherosclerosis between experimental and control animals is great. If, as is often the case, only a minor difference exists and the numbers of animals are relatively small, grading is not a suitable procedure, since it may lead to both false positive and false negative conclusions. Accordingly, it must be concluded that the experiments have failed to demonstrate that choline, even in large doses, has any effect whatever on either the development or the regression of experimental cholesterol atherosclerosis in the rabbit.

SUMMARY

A series of three experiments were undertaken to determine the effect of the oral administration of choline chloride on the development of experimental cholesterol atherosclerosis in the rabbit. It was concluded that the administration of moderate or large doses of choline to rabbits being fed cholesterol did not affect the development of atherosclerosis of the aorta or the content of lipids in the blood.

No effect of choline on the regression of experimental cholesterol atherosclerosis was detected in rabbits that had been fed cholesterol for three months and that had subsequently received 3 gm of choline daily for five months.

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PRODUCTION OF LIPOMATOUS AND ATHEROMATOUS ARTERIAL LESIONS IN THE ALBINO RAT

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THE RAT is generally considered to be very resistant to development of atheromatous arterial lesions.* Although numerous attempts to produce atherosclerosis in this species have been recorded, most have been completely unsuccessful, and the few reported instances of deposition of lipid in rat arteries have apparently had little resemblance to human atherosclerosis.†

The advantages of being able to use the rat as an experimental animal in the study of atherosclerosis are numerous. The similarity of its nutritional requirements to those of man, its short life span and small size, and its omnivorous appetite all contribute to its potential value as an experimental subject for this disease. Furthermore, knowledge gained by a study of the factors responsible for the development of atheromatous lesions in the rat may help supplement that gained by a study of the process in more susceptible species like the rabbit and chick, whose nutritional requirements and response to dietary cholesterol differ considerably from those of man. Even though the resistance of the rat to the deposition of lipid in its arteries may be greater than that of man, the conditions necessary to overcome this resistance may have application to the human disease process.

The study to be reported was undertaken with the purpose of observing the effects of prolonged feeding of various rations, with and without the added factor of an experimental hypertensive regime. It seemed unlikely, from the experience of other investigators, that nutritional manipulation alone would result in pathological arterial lipid deposition in the rat. Therefore, some of the animals of each diet group were subjected to a hypertensive regime in the hope that hypertension in the rat, as in the human, would increase the susceptibility of the animal to atheromatous change. The effects of these variables upon the concentrations of serum cholesterol and lipid phosphorus were determined during the course of the experiment and will be reported separately.[10] The histological effects upon the arteries of the rat were studied and will be described in this paper.

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* References 1 to 4.

† References 5 to 9.

PLAN OF EXPERIMENT

One hundred thirty-six middle-aged, obese, male rats were used in the experiment, simulating the age, sex, and nutritional state associated with a high incidence of atherosclerosis in man. The six synthetic rations employed were variations of a basic ration which was similar in carbohydrate, fat, and protein content to the average diet of four male patients who suffered attacks of coronary thrombosis between 35 and 50 years of age. The previous diet habits of each of these patients were reviewed and reconstructed by means of a careful diet history, and the probable daily percentage consumption of protein, fat, and carbohydrate was then calculated, using available tables of food composition. Cholesterol was added in an amount similar to the probable maximum cholesterol in American diets as calculated by Gertler and co-workers.[11]

Ten rats, selected at random from the 136, were killed at the beginning of the experiment to ascertain what pathologic processes, if any, were present in the group at this time. The remaining 126 rats were randomly distributed among six diets group as shown in Table 1.

The animals were continued on the previously mentioned rations for 14 weeks, at the end of which time three rats of each diet group were killed. At 20 weeks one-third of the surviving animals in each diet group (six rats) were then placed on a hypertensive regimen.[12] A second third of the animals served as injection controls, and the other six rats of each diet group were continued on diet only.

TABLE 1.—*Diet Groups*

Group No.	No. of Rats in Group	Daily Quantities of Variables in the Rations			
		Fat, Gm.	Choline, Mg.	Added Cholesterol, Mg.	Thiouracil, Mg.
I	21	2	125.0	62.5	0
II	21	2	12.5	62.5	0
III	21	6	125.0	62.5	0
IV	21	6	12.5	62.5	0
V	21	6	12.5	0	0
VI	21	6	12.5	62.5	25

All of the animals of each group were then continued on their respective rations for an additional period of about 20 weeks or until their death. Detailed autopsies were performed on all of the animals that died during the experiment and on those killed at the termination of the experiment.

MATERIALS AND METHODS

All of the 136 animals used were adult male albino rats of the Sprague-Dawley strain. They varied from 11 to 15 months of age at the beginning of the experiment and their average weight was 510 gm. (400 to 600 gm.). Prior to this experiment, all but one of the 136 rats had been used in previous dietary experiments, in which they had been fed a low protein ration[13] (diet 3E) for from 8 to 12 weeks followed by a short period of rapid protein repletion.[14] All had been fed Purina laboratory chow ad libitium for from 5 to 10 months before they were used for this experiment. They were kept in a thermostatically controlled room at 75 F., in large wire-bottomed cages with a maximum of seven rats per cage. They were fed weighed portions of diet each morning, the uneaten ration being weighed and discarded the following morning. Diet consumption records were kept throughout the experiment. The animals were weighed at weekly or biweekly intervals.

The composition of the rations used is given in Table 2. The four variables were fat, choline, cholesterol, and thiouracil (Table 1). Daily portions of the rations were comparable in calories, vitamins, protein, and minerals. Each week the diets were prepared by dissolving the cholesterol and the choline in the melted lard. These ingredients were then mixed with the dry ingredients. Except when the rats were being fed, the rations were stored in the dark at 4 C. in covered glass containers.

Hypertension in the rat was produced by the method described by Knowlton and co-workers.[12] This consisted of intravenous injections of 1.0 ml. of rabbit anti-rat-kidney serum given on two consecutive days. This was followed, four weeks later, by daily subcutaneous injections of

one milligram of desoxycorticosterone acetate (DCA) in sesame oil, and by the substitution of a 1% solution of NaCl for the drinking water. The injection controls were given 0.2 ml of sesame oil daily. This quantity of sesame oil was found to contain 2.6 mg. of a Lieberman-Burchard positive sterol, probably a phytosterol.

The anti-rat-kidney serum was produced by the method of Smadel.[16] A potent nephrotoxic serum was produced after three three-week courses of intraperitoneal injection of rabbits with ground perfused rat kidney. Preliminary observations disclosed that two 1 ml. intravenous injections of this serum given 24 hours apart resulted in a persistent, marked albuminuria with albumin content of 3 to 4 gm. per 100 cc., whereas the "normal" protein content in the urine of these rats was between 0.12 and 0.22 gm. per 100 cc. This dosage of anti-rat-kidney serum by itself produced a loss of weight but was not lethal over a two-month period, whereas two doses of 1.5 ml. given to three rats resulted in the eventual death of two of the animals.

Blood pressures were determined by the tail plethysmographic method of Williams, Harrison, and Grollman,[17] observing the precautions outlined by Sobin.[18] Determinations were made in

TABLE 2.—*Diet Composition*

Ingredients and Sources	Units	Diet Group Numbers					
		I	II	III	IV	V	VI
Vitamin test casein, General Biochemicals, Inc.	Gm.	15	15	15	15	15	15
Lard, Swift's Clover Leaf	Gm.	8	8	24	24	24	24
Dextrin (white), Merck & Company, Inc.	Gm.	67	67	31	31	31	31
Ruffex, Fischer Scientific Company	Gm.	4.25	4.7	4.25	4.7	4.95	4.6
Salt mixture*	Gm.	5	5	5	5	5	5
Choline chloride, General Biochemicals, Inc.	Gm.	0.5	0.05	0.5	0.05	0.05	0.05
Animal cholesterol, U. S. P., The Wilson Laboratories	Gm.	0.25	0.25	0.25	0.25	0.0	0.25
Thiouracil, Eli Lilly & Company	Gm.	0.0	0.0	0.0	0.0	0.0	0.1
Total	Gm.	100	100	80	80	80	80
Calories per gm.	....	4	4	5	5	5	5
Diet offered each rat each day	Gm.	25	25	20	20	20	20
Vitamins							
Thiamine HCl, Merck & Company, Inc.	Mg.	0.5	0.5	0.5	0.5	0.5	0.5
Riboflavin, Merck & Company, Inc.	Mg.	1.0	1.0	1.0	1.0	1.0	1.0
Nicotinic acid, Merck & Company, Inc.	Mg.	1.5	1.5	1.5	1.5	1.5	1.5
Pyridoxine HCl, Merck & Company, Inc.	Mg.	0.5	0.5	0.5	0.5	0.5	0.5
Ca Pantothenate, Merck & Company, Inc.	Mg.	1.5	1.5	1.5	1.5	1.5	1.5
2-methyl-naphthoquinone, Nutritional Biochemicals Corporation	Mg.	4.0	4.0	4.0	4.0	4.0	4.0
Percomorph oil, Mead Johnson & Company	Gtt.	0.5	0.5	0.5	0.5	0.5	0.5

* Hawk and Oser's modification of the Osbourne and Mendel salt mixture.[16]

triplicate on all rats at intervals of about four weeks during the last six months of the experiment. All determinations were made under light ether anesthesia and with the tail immersed in water at 40 C.

For purposes of determining the serum cholesterol and lipid phosphorus values during the experiment the rats of each diet group were divided into three approximately equal subgroups. Each of the animals was bled at monthly intervals from a lateral tail vein, 2 to 3 ml. being obtained from each animal. The blood from each subgroup was pooled equally. Within an hour after bleeding the serum was separated from the clot, and the protein constituents were removed from the serum by alcohol-ether precipitation and centrifugation. Serum cholesterol and lipid phosphorus values were determined by standard methods [19] adapted for smaller quantities of serum.

All animals were autopsied when they died or were killed during the experiment. Eighteen rats (three from each diet group) were killed at the 13th week, shortly before the hypertensive regime was instituted. Ninety-three rats survived 26 weeks or more, the majority of these being killed during the 42nd and 43rd weeks of the experiment.

All rats were killed by bleeding from the heart under ether anesthesia, the blood being collected for terminal individual determinations of serum cholesterol and lipid phosphorus.

All organs were examined grossly, and tissue samples of the following were fixed in neutral formalin-saline: heart (transverse and frontal blocks), entire aorta, lung, upper mediastinum, thyroid, liver, spleen and pancreas, mesenteric lymph nodes and blood vessels, stomach, adrenals, kidneys, testis, prostate and seminal vesicles, and bone marrow. Duplicate blocks were taken of aorta (both ends), liver, heart (transverse), adrenal, and, in some instances, of the kidney for preparation of frozen sections which were stained for neutral fat. Oil red O and scarlet red (Sudan IV) were used as lipid stains.

The remainder of the tissue blocks were embedded in paraffin and cut at 6μ. These were stained with hematoxylin and eosin. Additional sections of most of the kidneys were stained with Heidenhain's modification of Mallory's connective tissue stain and the periodic acid reticulum stain [20] to better demonstrate alterations of the glomerular tufts.

The aorta was treated specifically. After it was fixed, it was opened and examined. It was then rolled on itself with the intimal surface in, starting with the thoracic end. It was secured in the rolled position with a fine linen ligature, and imbedded on its side so that any section made through the block would pass through the entire length of the aorta.

EXPERIMENTAL RESULTS

In Vivo Observations (Body Weight, Food Consumption, and Blood Pressure).—The majority of the rats in the various diet groups showed a gradual increase in body weight throughout most of the experiment. There were two major exceptions to this. The animals receiving 25 mg. of thiouracil per day (Group VI) showed a sharp drop in body weight and diet consumption during the first six weeks of the experiment but this was reversed by a temporary reduction in the thiouracil intake to 6.25 mg. per day. There was a steady gain in body weight by this group thereafter even though the thiouracil intake was restored to 25 mg. per day at 23 weeks.

The six rats of each diet group which were given anti-rat-kidney serum, desoxycorticosterone acetate, and additional sodium chloride in their drinking water showed a steady decline in weight from the time of injection of the anti-kidney serum. This continued but did not seem to be accelerated when the desoxycorticosterone acetate and sodium chloride were begun two weeks later. The weight loss amounted to as much as 250 gm. per rat during the 20 weeks that this treatment was continued. Interestingly, it was not accompanied by any appreciable decrease in food consumption, suggesting that it resulted mainly from loss of nitrogen in the urine. The animals receiving the sesame oil injections showed no tendency to lose weight. At the end of the experiment the average weight of all of the rats not being made hypertensive was about 575 gm. with many individual animals weighing over 600 gm.

Figure 1 presents the weight trends graphically for one of the diet groups (Group IV, high fat, low choline). Although there were minor variations, essentially the same pattern of weight change was seen in the other diet groups.

As might be expected from the body weight changes, diet consumption was rather constant throughout the experiment. For the most part it varied between 60 and 80% of the quantity offered daily. The major exception was the group receiving thiouracil (Group VI) which showed a steady decline in dietary intake during the first seven weeks of the experiment.

Two blood pressure determinations about two weeks apart were made on all of the rats of each of the diet groups before hypertension was induced in the six rats of each of the diet groups. These two determinations on 103 rats showed an average systolic pressure of 118 with only four rats having a pressure above 150 mm. Hg on one or both determinations.

There was a general tendency for the systolic blood pressure of all groups to rise gradually during the experiment. However, the pressure rose more than 20 mm. Hg on one or more determinations in 17 of 27 rats (63%) observed for more than 45 days following the anti-rat-kidney serum injections and 23 of these 27 animals showed pressures greater than 150 mm. Hg on one or more determinations following the injections. On the other hand, only 12 of 55 rats (22%) receiving no anti-kidney serum or desoxycorticosterone acetate showed a rise of 20 mm. Hg and only 2 of these 55 rats developed pressures greater than 150 mm. Hg at any time during the experiment. Furthermore, the blood pressure rise was persistent or

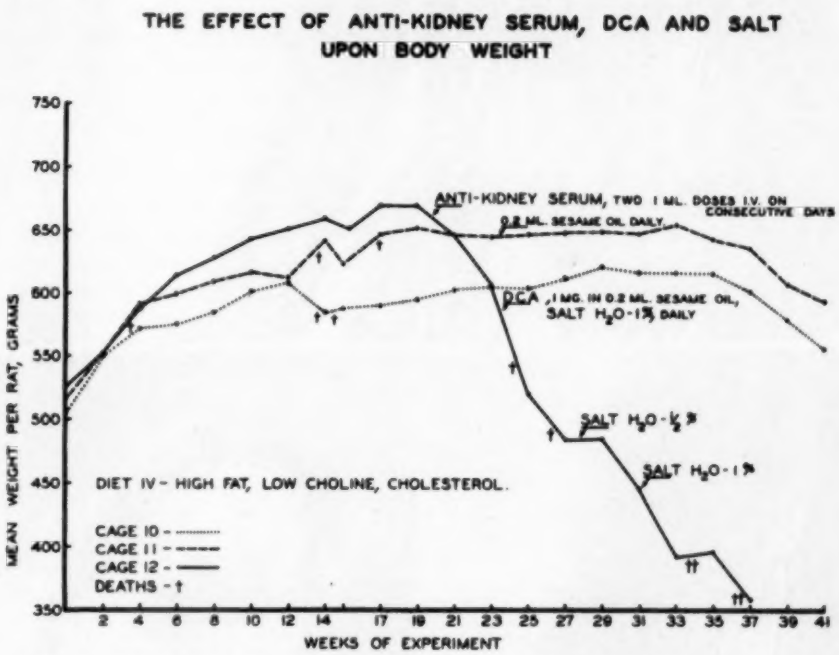

Fig. 1.—Effect of anti-kidney serum, desoxycorticosterone acetate (DCA), and salt upon body weight.

progressive in 8 of the 17 rats showing a rise of more than 20 mm. Hg while in none of the 12 animals of the other groups did the blood pressure remain elevated.

Histopathologic Observations.—No significant pathologic changes were noted in the tissues of the 10 rats killed at the beginning of the diet period. They showed no evidence of pneumonitis or nephritis, and no arterial lesions were observed.

The most noteworthy finding in the animals receiving the experimental rations was the presence of lipid in the walls of many of the coronary arteries. To a less degree this change was present in the renal arteries and aorta. For the purpose of clarity this will be called lipomatous change when the fat was deposited in the wall or endothelium of the vessels without being accompanied by any proliferative or by significant degenerative changes. It will be called atheromatous change when

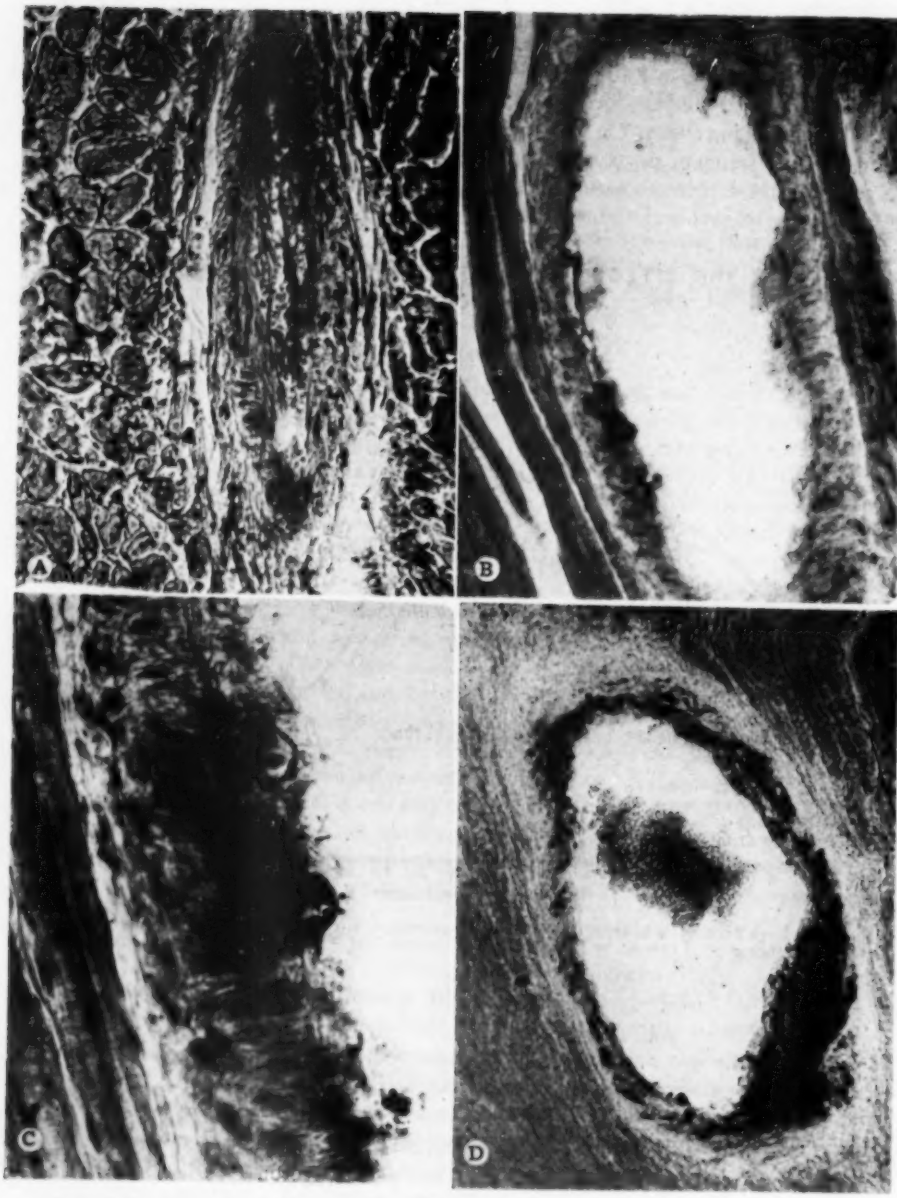

Figure 2

(See legends on opposite page)

the fatty deposition was accompanied either by intimal proliferation (plaque formation) or by definite degeneration of the artery wall or both. It seems probable that these two types of lipid deposition represent varying degrees of the same process, since the rats showing atheromatous coronary lesions usually had lipomatous lesions in nearby arteries. For tabulation purposes, rats showing visible lipid in one or more coronary arteries were termed positive. Of the 29 rats showing coronary lesions, 15 showed only lipomatous changes. These were usually confined to the smaller coronary branches, and consisted of more or less diffuse involvement of the intima and media by very fine droplets of lipid (Fig. 2*A*). The other 14 rats showing coronary lesions demonstrated atheromatous degeneration with some plaque formation in one or more vessels. The atheromatous changes were usually found in the larger arteries. Here even the "early" lesions showed rather large fat droplets deposited in or just under the intima (Fig. 2*B* and *C*), and were usually accompanied by focal fibrous thickening of the intima (Fig. 2*C*). In the more advanced lesions both the intima and media were usually involved with more intense atheromatous change of some parts of the wall than others, forming a distinct plaque (Fig. 2*D*). The most advanced lesions showed apparent narrowing of the lumen by a mixture of fibrous tissue, lipophages, and degenerated tissue (Fig. 3*A*) and could be easily recognized in hematoxylin-eosin stains (Fig. 3*B*). In two animals with these advanced lesions adjacent myocardial infarction was apparent, although no thrombus was present at the level of the section. No calcification or cholesterol crystals were seen. The Schultz-Lieberman reaction was positive on several of the atheromatous arteries.

Although the animals receiving the hypertensive regimen had a much higher incidence of coronary lesions, there was no difference in quality or intensity of the lesions in animals with this treatment, as compared to the rats receiving diet alone.

Although the over-all incidence of deposition of lipid in the aorta was about the same as the incidence of lesions in the coronary arteries, most of the aortic changes were slight. They consisted mostly of slight thickening and increased cellularity of the intima, with many of the cells containing lipid (Fig. 4). Only rarely was the media involved by visible lipid, and then only slightly. No atheromatous plaques were seen in the aorta.

The other instance in which lipid stains were employed in search of arterial atheromatous or lipomatous changes was in the renal vessels. Unfortunately, kidneys were preserved for fat stains only from the animals on the hypertensive regimen.

EXPLANATION OF FIGURE 2

Fig. 2.—*A*, Lipomatous change in a small coronary artery of a rat fed Diet II and subjected to the hypertensive regime and killed at 39 weeks. No arteritis was found in any of the tissues. Note the intimal and subintitimal localization of the lipid which is confined to one small segment of the wall and is distributed in very fine droplets. Frozen section, Sudan IV; × 340. *B*, Early atheromatous lesion in a large coronary artery of a rat fed Diet I and subjected to the hypertensive regime and killed at 41 weeks. No arteritis was seen in any of the tissues. Note the rather large droplets of fat localized in the slightly thickened intima. Frozen section, Sudan IV; × 255. *C*, Higher magnification of the involved portion of the same artery shown in 1*B*. The delicate intimal thickening is apparent as well as the extension of the fatty deposition into the inner portion of the media; × 960. *D*, Moderate atheromatous degeneration of a large coronary artery of a rat which receive1 Ration I, and was killed at 41 weeks. Here one sees extensive lipid accumulation in the intimal and subintimal areas with the formation of a larger plaque which involves more of the media and is covered by thickened intima. Frozen section, Sudan IV; × 235.

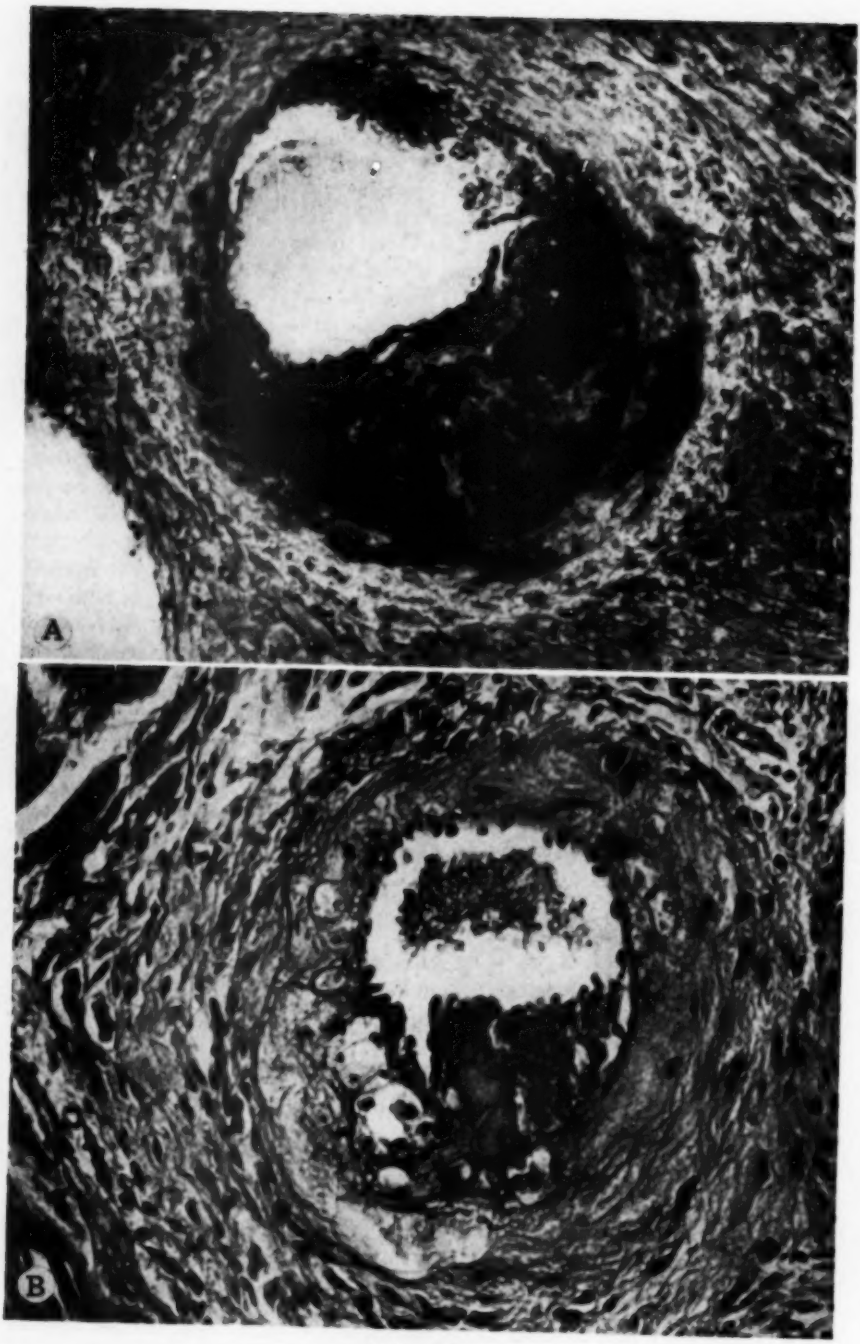

Figure 3

(See legends on opposite page)

Of the 28 animals in this category which survived more than 26 weeks only four showed visible lipid in the renal arteries. The true incidence may have been higher since many of the sections did not pass through the larger pelvic arteries. The lesions had the same characteristics as those in the coronary arteries.

Another site of lipid deposition in relation to vascular structure was in the glomerular tufts of the animals injected with the anti-kidney serum and desoxycorticosterone acetate. These rats uniformly showed an intense accumulation of lipid in the thickened basement membranes of all the glomeruli.

The only other vascular lesion observed was an arteritis which varied from an acute necrotizing process to a chronic fibrosing periarteritis. It was observed most frequently in the arteries of the pancreas, mesentery, kidney, and testis, and less frequently in the mediastinum, heart, and stomach. In the mesenteric vessels it was sometimes associated with aneurysm formation. In its acute form it resembled in all respects the lesions described by Selye and Pentz in unilaterally nephrectomized rats treated with desoxycorticosterone acetate and saline,[22] and in hypertensive animals described by Smith, Zeek, and McGuire.[23] In its milder and more chronic form, it resembled the lesions described in aging rats by Wilens and Sproul.[24]

These lesions were observed to a greater or lesser degree in 10 of 65 rats treated only with diet and surviving 26 weeks or longer, while 15 of 28 rats which were subjected to the hypertensive regimen and survived 26 weeks or longer showed a variable degree of arteritis.

There were two other pathologic processes which complicated the experiment. Most of the animals of each of the diet groups had a mild chronic bronchitis. In some instances this was accompanied by bronchiectasis and focal chronic pneumonitis. In spite of this high incidence of pulmonary infection, very few, if any, of the rats either lost weight or died from pneumonitis. The majority of the rats in all groups also showed a mild chronic interstitial nephritis at death, whether or not they had received anti-kidney serum. Although this lesion had many of the characteristics of a chronic pyelonephritis, this pathogenesis was not proved. The absence of an accompanying pyelitis made an ascending infection seem unlikely. These kidneys showed little or no evidence of glomerular disease as contrasted to those of rats receiving anti-kidney serum, and the lesion apparently had little, if any, effect on the general health of the animals. The chronic bronchitis and nephritis as well as the occasional occurrence of a generalized arteritis have been noted in other chronic experiments with Sprague-Dawley rats fed stock diets in other colonies.

EXPLANATION OF FIGURE 3

Fig. 3.—*A*, Advanced atheromatous degeneration in a large coronary artery of a rat fed Diet IV. This animal died 34 weeks after the beginning of the experiment. It had received anti-kidney serum and desoxycorticosterone acetate (DCA) and had a moderate elevation in blood pressure shortly before death. Autopsy revealed an enlarged heart with left auricular thrombosis, and both gross and microscopic evidence of infarction of the left ventricle. No arteritis was found in any of the tissues. The lesion shown here consists of a thickened atheromatous plaque which involves the proliferated intima, and, to a lesser extent, the underlying media. The endothelium extends over the plaque. Thrombosis could not be demonstrated. Frozen section, Sudan IV; × 330. *B*, Large subepicardial coronary artery of a rat fed Ration III for 37 weeks before it was killed accidentally while being bled. It had not been subjected to the hypertensive regime. Note the extensive focal degeneration of the thickened intima and underlying media. The endothelium covers the degenerated area. Many lipophages are seen intermixed with the atheromatous material. Paraffin imbedding, hematoxylin and eosin; × 425.

Apparently these lesions are characteristic of the older age period in this strain of rats, and are not the result of the experimental manipulation or colony conditions in this study.

The nephrotoxic nephritis observed in the animals receiving anti-kidney serum was similar to that described by others.‡ The kidneys were usually markedly enlarged, finely granular, firm, and pale. Microscopically they showed enlargement of all glomeruli with marked thickening of the basement membrane, numerous

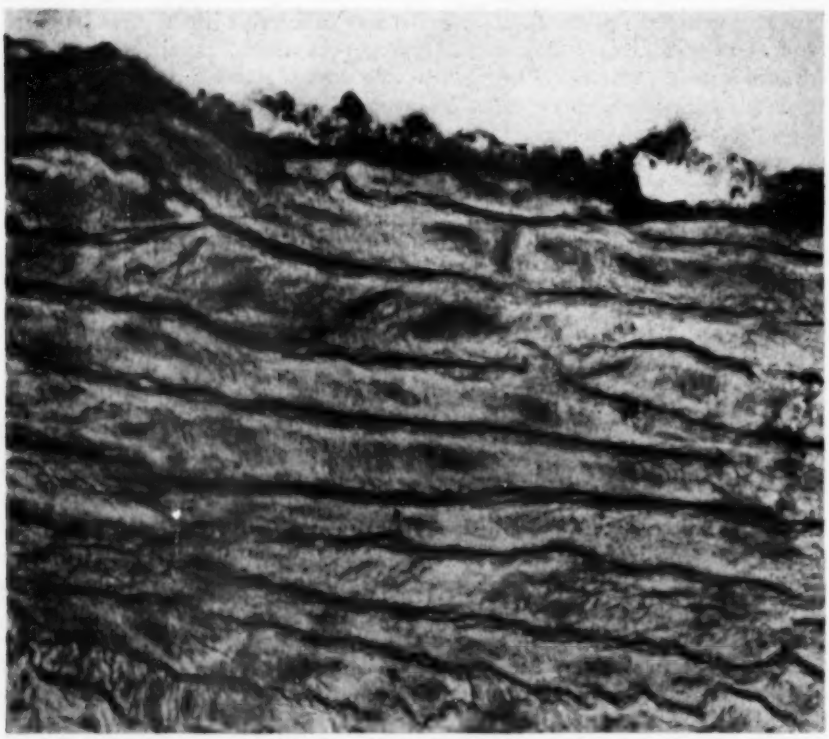

Fig. 4.—Aorta of a rat fed Diet VI with thiouracil and killed at 41 weeks. The small fat droplets are deposited in the slightly thickened intima but no plaques were formed and there is no evidence of degenerative change. Frozen section, Sudan IV; × 460.

synechiae, occasional crescent formation, variable tubule atrophy, and hyaline eosinophilic precipitate in tubules.

Some degree of fatty change was seen in all of the livers, being much greater in the animals receiving less choline in the diet and in the animals receiving the higher fat rations. No cirrhosis or other evidence of liver disease was observed.

The thyroid glands of the animals receiving thiouracil were markedly and diffusely hyperplastic. In addition, three rats in Diet Group II (low fat, low choline) showed what appeared to be early but rather undifferentiated neoplasms of the

‡ References 24 to 27.

thyroid gland. These had the cellular characteristics of carcinomas, being made up of small pleomorphic cells arranged in cords. The nuclei were hyperchromatic and showed numerous mitotic figures. The borders of the nodules were irregular but there was no evidence of invasion or metastasis outside the gland.

The Factors Influencing Coronary Lipid Deposition.—The incidence of coronary lesions in each of the diet groups, with or without the hypertensive regimen, is summarized in Table 3. For the purposes of tabulation lipomatous and atheromatous lesions have been considered together. Since no animal showed lesions unless it had been fed one of the experimental rations for a minimum of 26 weeks, data for only those animals surviving for this period or longer are considered in the tabulation

TABLE 3.—*Lipomatous and Atheromatous Lesions in Coronary Arteries*

	Diet Alone			Diet Plus "Hypertension"			Totals		
Group No.	No. Surviv. 26 Wk.	No. with Lesions	% with Lesions	No. Surviv. 26 Wk.	No. with Lesions	% with Lesions	No. Surviv. 26 Wk.	No. with Lesions	% with Lesions
I	9	3	33.3	5	2	40.0	14	5	35.7
II	11	2	18.2	4	3	75.0	15	5	33.3
III	11	7	63.6	3	0	00.0	14	7	50.
IV	10	0	0	5	2	40.0	15	2	13.3
V	12	0	0	5	5	100.0	17	5	29.4
VI	12	2	16.6	6	3	50.0	18	5	27.8
Totals ..	65	14	21.5	28	15	53.6	93	29	31.2

TABLE 4.—*Effect of Time upon the Development of Fat-Containing Lesions of the Coronary Arteries*

Weeks After Experiment Started	Total Deaths*	No. of Rats with Lesions
0- 8	0	0
9-16	20†	0
17-26	6‡	0
27-36	18‡	10
37-44	75§	19

* Includes only the rats in which histological specimens were obtained.
† Eighteen rats killed at 13 weeks.
‡ Spontaneous deaths.
§ Majority killed.

(Table 4). The injection of sesame oil in one-third of the animals of each diet group had no consistent influence upon the incidence of coronary fatty lesions. Therefore, these animals have been tabulated with those receiving diet alone. The animals subjected to the hypertensive regimen have been tabulated separately, since they exhibited an over-all incidence of coronary disease which was approximately twice that of the animals with only nutritional variables.

In addition to the marked increase in over-all incidence resulting from the hypertensive regimen, other trends were noted. Although there was no significant correlation between the incidence of coronary lipid deposition and the amount of fat in the diet in the first four groups of animals treated with diet alone, there was a significant correlation between the incidence of lesions and the amount of choline fed, more lesions occurring in the groups fed a high choline ration (Fig. 5). This trend

appeared to be reversed when the hypertensive regimen was added to the dietary treatment, and under these conditions there was evidence that lesions occurred more frequently on a low fat diet. The dietary administration of thiouracil did not have any appreciable influence on the incidence of coronary lesions. In contrast to this the lipomatous aortic lesions were most marked and the incidence of aortic lipid deposition was greatest in animals receiving thiouracil in their ration. It is interesting that the high fat, low choline ration without added cholesterol (Group V) resulted in no coronary lesions in 12 animals and that all five of the rats receiving this ration and the hypertensive regimen showed lesions. Further study will be necessary to confirm this finding.

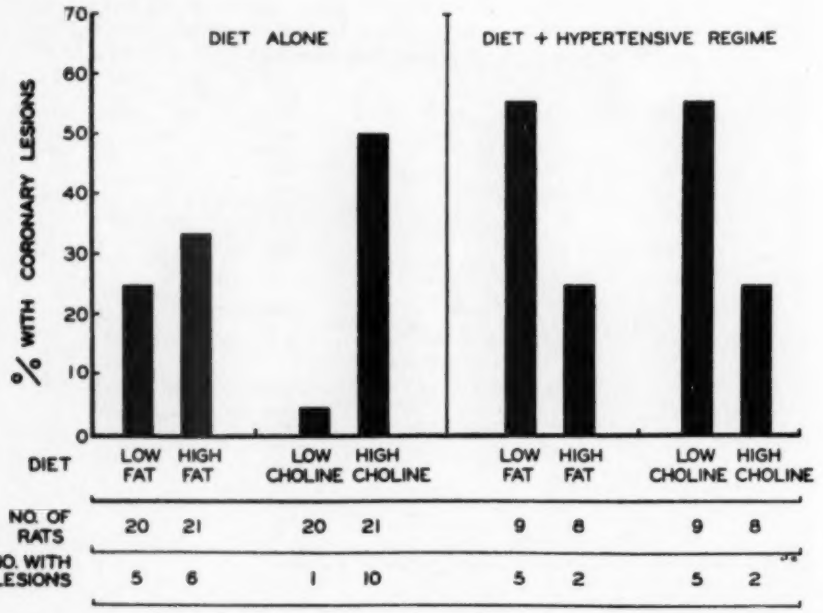

Fig. 5.—The effect of low and high levels of dietary fat and choline upon the incidence of lipomatous and atheromatous coronary lesions in the coronary arteries of rats fed Rations I to IV and surviving 26 weeks or longer.

The maintenance of obesity did not appear to be an important predisposing factor in the development of the coronary fatty lesions (Fig. 5). In fact, the rats subjected to the hypertensive regimen had a profound weight loss (Fig. 1) and yet showed the highest incidence of lesions (Table 3). The results do not eliminate the possibility that initially well-filled fat depots may have been an important prerequisite to the development of lesions.

Although there appeared to be a correlation between the presence or absence of either the hypertensive regimen or resulting "hypertension" and the incidence of coronary lipid deposition (Fig. 6), neither hypertension nor the administration of anti-kidney serum and desoxycorticosterone acetate with salt were necessary for

lesions to develop. Since, in general, the animals subjected to this treatment also had elevated serum cholesterol values accompanied by high cholesterol : lipid-phosphorus ratios,[10] it seems likely that abnormalities in lipid metabolism rather than mechanical factors were responsible for the marked increase in incidence of coronary lesions in these animals. In keeping with this suggestion is the fact that there was a less definite correlation of actual observed hypertension with the incidence of coronary lesions as compared with the more definite correlation between the instituting of the hypertensive regimen and the incidence of lesions (Fig. 6).

Although coronary lipid deposition was more frequent in the animals showing arteritis (Fig. 6), there are reasons to doubt that there was any cause-effect rela-

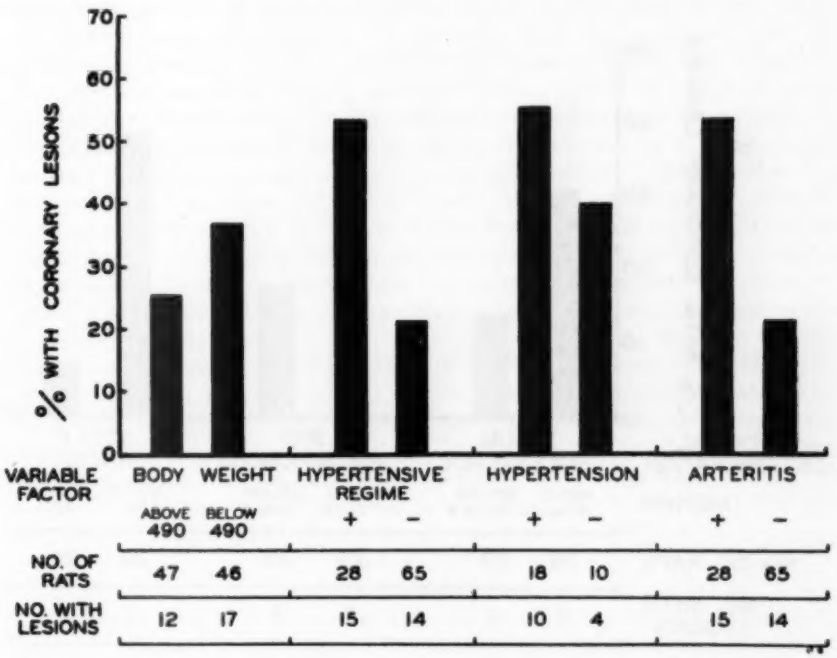

Fig. 6.—The effect of certain experimental variables including the presence of arteritis upon the incidence of lipomatous and atheromatous coronary artery lesions in the 93 rats surviving 26 weeks or longer.

tionship between these two types of arterial lesions. The arteritis was almost always confined to small and medium-sized arteries, while the lipid-containing lesions often involved large renal and coronary arteries. Most of the arteries showing visible fat in their walls showed no evidence of arteritis. Furthermore, previous experience with a large group of ald male Sprague-Dawley rats fed stock diets has revealed many animals with arteritis but none with coronary fatty lesions.[28] Perhaps of greater significance is the fact that in the present experiment 14 of the rats showing definite coronary fatty lesions, some of them very marked, showed no evidence of arteritis in any of their tissues (Fig. 6). It is quite possible, however, that arterial

injury of less degree than that necessary to produce arteritis may have helped predispose the vessels to lipid deposition. Nor can one be certain that arteritis was not present at some earlier time in the vessels showing lipid deposition at death.

The time-interval for which the animal had received the experimental rations was evidently an important factor in determining whether or not coronary lipomatous or atheromatous lesions appeared (Table 4), but the total age of the animal appeared to be less important. Birth dates were available on 91 of the 93 rats which survived 26 weeks or longer. Of the 49 rats which were younger than 21 months at the time of death, 15 showed lesions, while 14 of the 42 rats which were between 21 and 25 months of age at death showed coronary lesions.

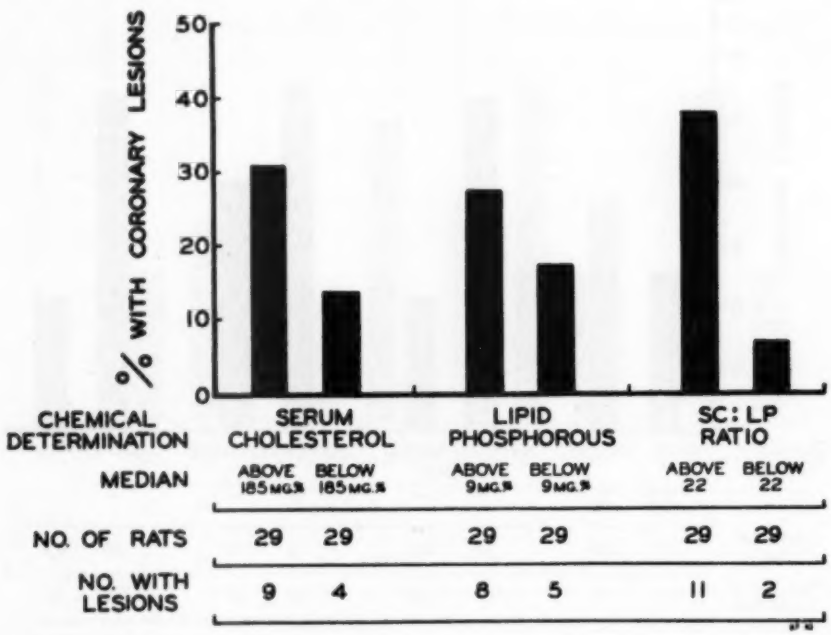

Fig. 7.—The correlation between terminal blood chemical findings and the incidence of coronary lipomatous and atheromatous lesions in the 58 rats in which these determinations were made during the 42nd and 43rd weeks of the experiment.

An association of the presence of coronary fatty lesions with abnormal blood lipid values at the time of death is shown in Figure 7. Serum cholesterol and lipid phosphorus determinations were obtained on 58 of the rats at the time they were killed; of these, 13 had coronary lesions. There was a tendency for animals showing an elevation of either the serum cholesterol or the lipid phosphorus to have a higher incidence of coronary lesions (Fig. 7). The statistically significant correlation was between elevation of the serum-cholesterol : lipid-phosphorus ratio and the presence of coronary lipid. Of the 29 rats with a ratio above 22, there were lesions in 11, while lesions were present in only 2 of the 29 rats with a ratio below 22.

Statistical Analysis.—The significance in the differences of the incidence of the fatty coronary artery lesions among the various diet groups was evaluated from two by two contingency tables using the χ-square test with one degree of freedom, the Yates correction being applied when indicated. Comparison of the differences in incidence among the animals subjected to dietary manipulation only (Table 3), revealed that the differences in incidence between Groups III and IV and between Groups III and V were significant to the 1% level or better. The differences in incidence between Groups II and III and Groups III and VI had a likelihood of chance occurrence of less than 10 times in 100, but more than 5 times in 100. When the incidences of coronary artery lesions of the rats receiving the diet plus the hypertensive regimen and those receiving diet alone were compared for each diet group, the only groups showing a clearly significant difference in incidence by the χ-square test were those receiving Diet V. On the other hand, on an over-all basis, the difference in coronary fatty lesions between the animals subjected to the hypertensive treatment and those receiving diet alone had a χ-square value of 7.03 with a probability of reproducibility of 99%. Similarly, a comparison of the incidence of lesions among the animals receiving a low choline intake versus those on high choline rations (first four diet groups) revealed that the differences in incidence would occur by chance less than 1 time in 100. On the other hand, the differences in incidence between the high and low fat diets in these same groups was not significant and variation of either fat or choline did not produce a significant change in incidence in the rats on the hypertensive regimen. Of the other lesions observed, the correlation of arteritis with the presence of atheromatous lesions was significant for the entire experimental group but not for any individual diet group. Nephritis or bronchitis was not present or absent in a significant number of animals with lipid in their coronary arteries. In the animals on which terminal blood-chemical determinations were made, neither the serum cholesterol nor the lipid phosphorus elevation was related significantly to the presence of coronary fatty lesions. A ratio of serum cholesterol to lipid phosphorus above 22 correlated well with the incidence of atheromatous or lipomatous lesions resulting in a χ-square value of 6.34 which indicated that this relationship would occur by chance only 2.5 times in 100.

COMMENT

This study differs from previous recorded attempts § to produce atheromatous lesions in the rat in several respects. The animals were older and more obese at the beginning of the experiment than in most of the earlier investigations. The rats were fed the experimental rations for a longer period than many have employed.

Few previous studies have utilized synthetic diets similar to the ones which these animals received. Many of the older recorded experimental diets consisted of cholesterol mixed with a stock ration which consisted largely of cereals. In many instances only vegetable oils were added as the fat source. More recent experiments in this laboratory suggest that the source of the dietary fat is important and probably has as much influence upon the development of coronary lesions as the addition of cholesterol.[39]

The coronary arteries, which exhibited the most significant lesions in this experiment, have been studied by means of stains for neutral fat in less than half of the

§ References 5 to 9 and 29 to 38.

previously reported studies. This is probably important, since only the more advanced lesions are visible in the hematoxylin and eosin stains.

Few investigators have utilized an additional stress, comparable to the hypertensive regimen employed in this experiment, in an attempt to increase the susceptibility of the arteries to fatty deposition. In one experiment where exercise was added to dietary manipulation, lesions were observed.[7] While it appears probable that the administration of the nephrotoxic serum and desoxycorticosterone acetate exerted their main effects by altering lipid metabolism rather than by subjecting the arteries to a higher systolic pressure, there can be little doubt that this additional experimental procedure markedly increased the incidence of the lesions.

It is difficult to evaluate the effect of the period of protein-depletion to which these animals were exposed some time before this experiment began. Although a more recent study indicates that a preceding period on a low protein ration is not necessary to produce mild lipomatous lesions in similar or greater incidence,[39] it may have increased the severity of the lesions in this experiment. Acute experiments in this laboratory have shown that a low protein ration will accelerate the rate at which the cholesterol : lipid-phosphorus ratio is elevated by dietary means in the rat.[40] In interpreting the results of the present experiment it should be remembered that the rats subjected to the hypertensive regimen were probably protein-deficient since they suffered severe weight loss, and showed marked proteinuria after the administration of the nephrotoxic serum.

Hartroft and co-workers [41] have recently reported the occurrence of similar lipid-containing coronary artery lesions accompanied by severe atheromatous and calcific aortic lesions in rats which developed cirrhosis after being fed choline-deficient rations which were high in lipid and poor in protein for long periods of time. This is difficult to reconcile with the results of this study since, among the rats with only nutritional manipulation in the experiment reported here, the highest incidence of lesions was in the group receiving a relatively high choline-intake. High choline levels have been reported to augment chicken atherosclerosis [42] and monkey atherosclerosis [43] under certain experimental conditions. None of the rations in the present experiment were choline-free, and no cirrhosis was produced, but it is evident from this study that choline deficiency is not the only dietary prerequisite to atheromatous lesions in the rat. Further study will be necessary to ascertain the role which choline and the other dietary variables play in the deposition of lipid in the arteries of rats.

It seems apparent from the recent study recorded by Page and Brown,[9] that prolonged hypercholesterolemia by itself does not result in atheromatous lesions in the rat. The factors of age, preexisting obesity, and low-grade injury to arteries, too slight to result in arteritis, must be considered in future studies of the reaction of this resistant species to hypercholesterolemia. In this experiment the highest levels of blood cholesterol were found in the rats subjected to the prolonged administration of thiouracil,[10] but the coronary artery lesions were not particularly predominent in this diet group.

In spite of the multiplicity of the questions raised by this experiment and the other studies cited, it appears important to continue to investigate atherogenesis in the rat in the hope of understanding the reasons for its partial physiologic protection from atherosclerosis. It seems unlikely that it is either anatomical or the result of an inherently different tissue-reaction to cholesterol as has been suggested.[11] The

dichotomy between the severity of the coronary and aortic lesions in this experiment is consistent with phenomena recorded in experimental atherogenesis in other species [4] and it has been observed in man.‖

The correlation of the elevation of the cholesterol : lipid-phosphorus ratio with the incidence of the atheromatous change is of considerable importance since it adds another species to those in which this relatively simple chemical determination gives some indication of the probabilty of development of atheromatous lesions.¶

SUMMARY

One hundred twenty-six middle-aged, obese, male rats, divided into six groups of 21 rats each, were fed six synthetic rations in which fat, choline, cholesterol, and thiouracil were varied. One-third of the rats of each diet group was subjected to a hypertensive regimen consisting of two injections of a potent nephrotoxic serum followed by daily injections of desoxycorticosterone acetate, and salt in the drinking water.

Tissues were studied from 93 of the rats which survived 26 weeks or longer. Thirty-one per cent of these survivors showed fatty deposition in one or more of their coronary arteries. In 15 of these rats the change consisted entirely of small quantities of visible lipid deposited focally in the intima and media of coronary arteries without intimal proliferation or degeneraton of the wall. In 14 of the rats definite plaques were found, usually in larger coronary vessels, and frequently accompanied by degeneration of the tissue of the plaque and the underlying media. Similar changes were observed in a small number of renal arteries. The aorta showed a slight fatty deposition in the intima of 30% of the rats, but this was never accompanied by true atheromatous change.

The animals subjected to the hypertensive regimen had two times the incidence of lipid-containing coronary lesions compared to those limited to dietary manipulation, but there was no significant correlation between the presence of observed hypertension and the development of lesions. There was also a high incidence of arteritis in the rats on the hypertensive regimen, but it seems likely that the fatty lesions were associated with abnormalities in lipid metabolism which these animals showed, rather than the arteritis. Of the rats receiving diet alone, those eating the rations containing the higher choline concentrations had a significantly higher incidence of lesions than those fed the lower choline levels. No significant relationship could be established between the incidence of the coronary lesions and the degree of obesity, the presence of hypertension, or the higher levels of fat in the diets. A significant correlation was observed between the presence of a terminal elevation of the serum-cholesterol : lipid-phosphorus ratio and the incidence of lesions.

Statistical evaluation of the data was performed by William Allen under the supervision of Dr. Leonard J. Savage of the Committee on Statistics of the University of Chicago.

The photomicrographs were taken by Mr. Jean Crunelle, chief photographer, The University of Chicago Clinics.

Miss Barbara Ruby, medical nutritionist of The University of Chicago Clinics, obtained the diet histories and made the calculations.

Dr. Charles Reiner and Dr. George Gomori prepared the sections stained with the Shultz-Lieberman technique.

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¶ References 46 to 49.

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OBSERVATIONS ON THE LOCALIZATION OF ALKALINE PHOSPHATASE IN HEALING WOUNDS

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THE PRESENCE of alkaline phosphatase activity in healing skin wounds of rats was first described by Fell and Danielli.[1] They found evidence of enzymatic activity in the cytoplasm and nuclei of fibroblasts and associated with newly-formed connective tissue fibrils. Although these observations have been repeatedly confirmed in the skin,* observations on fibroplasia at other sites have not always shown the association of phosphatase activity with fiber formation.† The hypothesis that this enzyme is in some way concerned in fiber formation is therefore open to doubt.

In the course of some experiments on the connective tissue of healing skin wounds we were impressed by the occurrence of the most intense alkaline phosphatase activity in the vicinity of regenerating epithelium. This suggested that the regeneration of epithelium might determine the presence of phosphatase activity rather than fiber formation. In order to test this hypothesis a series of observations were made on the localization of phosphatase activity in relation to epithelial implants introduced into deep and superficial wounds.

EXPERIMENTAL PROCEDURES

The experimental animals were 3- to 4-month-old female albino rats of the Sprague-Dawley strain. The hair of the back was clipped with electric clippers and shaved with a razor. In the first experiment, deep wounds were made in seven animals as follows: The skin was incised in the midline and the muscle exposed in the interscapular region. Two deep punch wounds in the muscle on either side of the midline were made with a 6 mm. cutaneous punch, and the muscle core removed. A 4 mm. circle of shaved skin was removed from a donor site and inserted, epithelial surface downwards, into one of the wounds; the other wound was allowed to fill with blood. The surface wounds were then closed with clips. The animals were killed at intervals of 1, 2, 4, 6, 9, and 14 days after wounding.

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In the second experiment a series of six subcutaneous wounds, each containing an epithelial implant, was made on the back of two animals. Each wound was made with a 6 mm. punch to remove an epithelial core, which was inverted and slipped under the skin before closing the opening with a clip. The wounds in each animal were made 14, 9, 6, 4, 2, and 1 days before the day on which the animals were killed.

The wounds were excised and the tissues fixed in cold acetone, impregnated with 4% collodion and embedded in paraffin in vacuo. Sections were prepared at 5 μ and stained as follows: (1) for alkaline phosphatase, using both the CaCoS and the azo-dye methods as described by Gomori;[7] (2) for glycogen, using the alcoholic periodic acid-Schiff method of Hotchkiss;[8] (3) for multiple tissue components, using Gomori's modification of the trichrome stain.[9]

Fig. 1.—Section of six-day deep muscle wound containing skin implant. Acetone fixation, paraffin imbedded, 5 μ, CaCoS technique for alkaline phosphatase, incubated six hours. Note the dense staining adjacent to the proliferating epithelium of the cyst wall. Staining of areas adjacent to the injured muscle is also evident ($\times$ 20).

OBSERVATIONS

The wounds healed well without evidence of infection. There was epithelial proliferation from the cut edges of the skin implants which resulted either in the formation of epithelial cysts or in the production of tongue-like epithelial outgrowths. This newly formed epithelium was always rich in glycogen as shown by the periodic acid-Schiff technique. Alkaline phosphatase activity was maximal on the sixth postoperative day in all the wounds. In the wounds containing the

implants, there was intense activity in the connective tissue subjacent to the proliferating epithelium (Fig. 1), regardless of whether this occurred deep in the muscle or in the subcutaneous zone. Comparable areas of granulation tissue in deep muscle wounds without epithelial implants had cystic cavities containing serous fluid. There was no phosphatase activity adjacent to the lumen of these cavities (Fig. 2). In the subcutaneous wounds the distribution of phosphatase activity in the connective tissue was clearly altered by the presence of the grafts. Staining often ended quite sharply at the free edges of epithelial projections. Zones of newly formed connective tissue away from the epithelium, although comparable morphologically to the sites of intense activity, remained unstained.

Fig. 2.—Section of six-day deep muscle wound without skin implant. Preparation and staining identical with section shown in Figure 1. Note central cystic cavity lacking epithelium and without phosphatase activity in adjacent new connective tissue. Staining of capillaries in muscle and in new connective tissue is evident. Dense areas of staining are adjacent to new muscle buds and fresh capillaries (× 20).

In the deep wounds, phosphatase activity was also seen in the marginal zones of the wounds where the granulation tissue was in contact with regenerating muscle (Figs. 1 and 2). In these areas the capillaries stained intensely and there was also some staining of the connective tissue fibrils and fibroblasts. This staining faded off a few cell-widths from the highly active capillary and muscle sites. The distribution of phosphatase activity in the wounds was seen as essentially the same with the CaCoS and the azo-dye techniques.

COMMENT

The observation that alkaline phosphatase was present in the fibrous component of healing skin wounds led to the supposition that the enzyme might play a role in collagen fiber formation.[1] Some support was given to this hypothesis by the diminution in phosphatase activity observed in healing wounds of scorbutic guinea pigs in which fiber formation is known to be impaired.[2] Against this supposition is the fact that there is no evidence that phosphatase inhibitors will impair wound healing.[10] The hypothesis becomes untenable, as pointed out by Robertson and co-workers,[6] when it can be shown that in certain sites normal fiber formation occurs without any demonstrable phosphatase activity.

It is necessary, therefore, to find an alternative explanation for the undoubted rise in phosphatase activity which occurs in healing skin wounds. It has been shown by Gold and Gould,[11] that when collagen fibers are reconstituted in vitro from rat-tail tendon in the presence of alkaline phosphatase there is some incorporation of enzyme into the newly-formed fibers. This has suggested that the localization of phosphatase in healing wounds may depend in part upon its absorption from the tissue fluids, and thus bear no direct relationship to the metabolism of connective tissue. This theory still does not explain why phosphatase should be absent at some sites of fibroplasia and present in others, unless there are local factors which determine the concentration of the enzyme in tissue fluid.

The present observations suggest that the high local concentration of phosphatase in healing wounds is in some way related to the metabolic activity of epithelium and possibly skeletal muscle. Huggins [12] has shown that a high concentration of phosphatase can be induced in fascia by implants of bladder mucosa. When these preparations are examined for enzyme histochemically, they show a very striking resemblance to the appearance seen when skin epithelium is implanted. Gomori [13] has suggested the possibility that bladder epithelium has a specific inductive power in certain kinds of connective tissue resulting in the proliferation of phosphatase producing fibrocytes. A similar theory would account satisfactorily for the histochemical appearance in healing wounds, particularly since one sees no evidence that the epithelial cells produce the enzyme themselves. In view, however, of the apparent absorptive capacity of growing connective tissue for phosphatase,[11] the possibility remains that the enzyme is not produced by fibroblasts at all, but is adsorbed onto the connective tissue from some other tissue component.

SUMMARY

The observations presented indicate that, contrary to early beliefs, histochemically demonstrable alkaline phosphatase is not associated primarily with connective tissue formation in healing wounds. The presence of this enzyme in new fibers in the experiments described is found to be related to the presence of other proliferating tissue, particularly regenerating epithelium. The mechanism of incorporation and source of enzyme is still not clear. The possible causes of the presence of alkaline phosphatase in some sites of wound healing are discussed.

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REGIONAL ENTEROCOLITIS IN COCKER SPANIEL DOGS

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SINCE the pioneering work of Crohn, Ginzburg, and Oppenheimer [1] in 1932, regional ileitis (regional enterocolitis, cicatrizing enteritis) has been recognized as a clinical and pathologic entity in the human. Both acute and chronic phases exist, and there are adequate pathologic criteria for the diagnosis.

In the veterinary medical literature recent studies indicate that in swine regional or terminal ileitis has long been observed as an incidental finding, particularly by meat inspectors. From Denmark, Adsersen [2] reported 201 cases of the disease in 4,096 inspected swine. A fundamental work on terminal ileitis in swine was published in 1951 by Emsbo,[3] who established it by a distinctive pathologic-anatomic appearance to be a pathologic entity. The possibility of a fundamental conformity between porcine and human regional enteritis was pointed out. In swine the disease was sharply limited to the terminal ileum, but in far advanced cases it evidently spread into the cecum and colon. Emsbo's article, establishing regional ileitis as an entity in swine, is comparable to the pioneering in the human disease by Crohn and associates.

Very little has been reported concerning this disease in dogs. Similar inflammatory intestinal processes in dogs are mentioned, without particular emphasis or investigation, in the textbooks of Hutyra, Marek, and Manninger [4] and Nieberle and Cohrs.[5] Only the experimental report of Reichert and Mathes [6] exists describing the production of this disease in dogs. They cannulated intestinal lymphatics and injected sclerosing solutions. A lymphedema was produced, with filling and blocking of the lymphatics by large pale cells and with ensuing chronic lymphedema after one month. Later only a lymphocytic and mononuclear infiltrate persisted. The intestinal muscular and submucosal layers were most notably thickened, and the mucosa was not ulcerated. The changes were enhanced by intravenous injections of Escherichia coli bacilli. Grossly and microscopically the changes closely reproduced the pathologic picture of human regional or cicatrizing enteritis.

The experiments of Chess and associates,[7] with use of sand and talc in isolated ileal loops and as part of the diet of dogs, did not appear to simulate regional enteritis production so closely. Reichert and Mathes discarded the use of crystalline

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silica injections as too abrasive. Feeding experiments in about 20 rats and 12 guinea pigs, using silicates or beryllium salts with or without high fat diets, have failed to produce any intestinal disease.* In veterinary practice it is common to see dogs with histories of habitual ingestion of dirt and sand. At autopsy such animals have this foreign material in the gastrointestinal tract, with only slight superficial irritation. It appears doubtful that such agents have any etiologic relationship to regional enterocolitis, since otherwise this condition in dogs would be relatively common.

Spontaneous cicatrizing or regional enterocolitis is seemingly rare in dogs, since there is no report of the disease in the literature. At the Angell Memorial Animal Hospital, where approximately 500 autopsies annually are performed on dogs and cats, two cases were seen during the past year.*

REPORT OF CASES

Case 1 (S52-203, A52-230).—A black cocker spaniel, male, 4 years old, had been sick for approximately six weeks, starting with violent vomiting. Later there was hemorrhagic vomitus and bloody diarrhea. A local veterinarian treated the animal, without improvement.

Three weeks later, on admission to Angell Memorial Animal Hospital, x-rays demonstrated thickening of the cecum, colon, and rectum (Fig. 1). The dog was placed on a bland diet, and sedatives were administered for nervousness. He improved slightly, but bloody diarrhea continued and progressed with serious bleeding.

An exploratory laparotomy was performed, which revealed the cecum, part of the colon, and the rectum to be inflamed, thickened, and firm. A specimen was taken for biopsy. The dog was killed the same day, and an autopsy was performed.

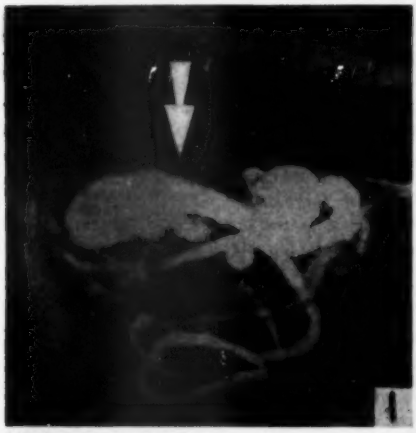
Grossly the abnormal findings at autopsy were limited to the colon, rectum, and regional lymph nodes. The ileum appeared normal. The cecum was enlarged, and the wall thickened to 0.4 cm. Its mucosa was spotted with reddened areas over the entire surface. The colonic thickening extended for 15 cm. distal to the ileocecal valve, with mucosal hemorrhages similar to those described in the cecum. In the rectum, 5 cm. above the anus, there was a comparable thickening 5 cm. long. The blood vessels in the affected areas were slightly dilated, and the regional lymph nodes were enlarged.

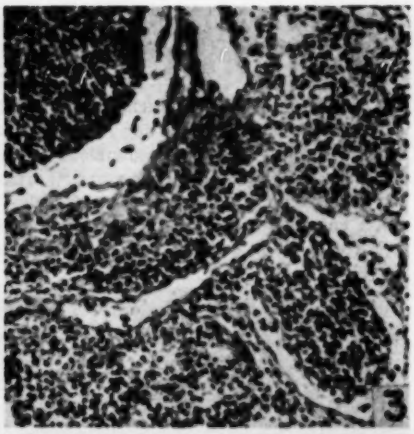
Microscopically both the tissue taken for biopsy from ileocecal valve and the autopsy specimens from affected colon showed the same abnormalities. There was a striking thickening of intestinal submucosa by edema and a leucocytic infiltrate composed of polymorphonuclear leucocytes, eosinophiles, and a few macrophages. With Giemsa's stain, mast cells were also identified in the inflammatory exudate. The infiltrate extended superficially into the lamina propria. Mucous membrane glands were generally intact but with local attentuation or degeneration.

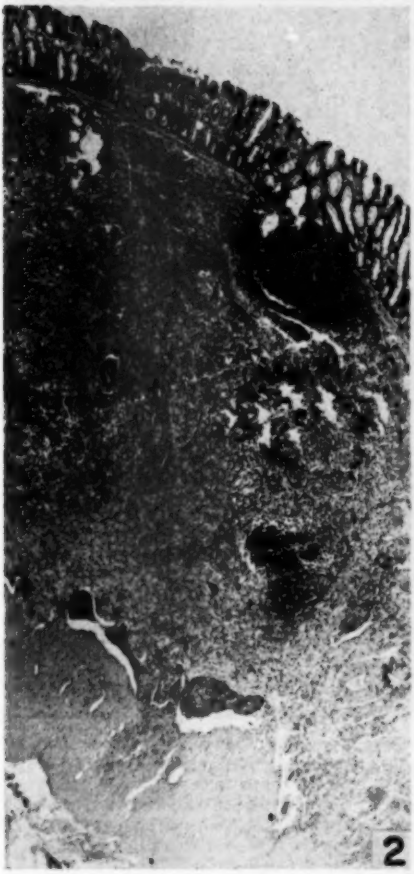
Lymphatics in the intestinal wall were prominent, and in the lumens of several there were leucocyte nodules, particularly in lacteals at the boundary of submucosal and muscular layers (Fig. 2). The intralymphatic leucocytic aggregates merged with early granulomas, containing foreign body giant cells in two instances. Similar intralymphatic leucocyte masses were evident in mesenteric lacteals and in capsular sinusoids of the regional lymph nodes (Fig. 3). The lymph nodes also showed hyperplasia, with focal acute and chronic inflammation and necrosis.

Microscopic sections of liver, adrenal gland, and thyroid were negative. Splenic sinusoids demonstrated acute congestion. There were foci of chronic inflammation in the kidney. Bone marrow was hyperplastic, with numerous eosinophilic myelocytes.

* A third similar case in a black cocker spaniel dog was previously observed by one of us (Petrak) as a student in Philadelphia.







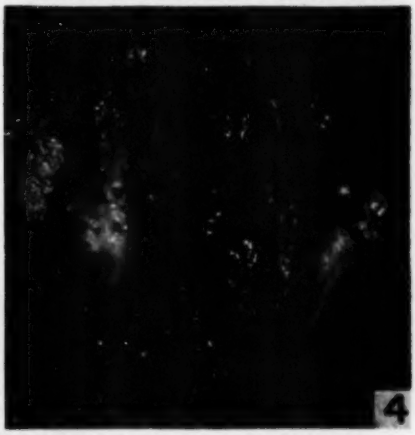


Fig. 1 (Case 1).—X-ray study, with barium by mouth, of the intestine. The arrow indicates the ascending colon, which has a thickened wall and serrated luminal outline. The fainter feathery white area at left margin represents residual barium in the stomach.

Fig. 2 (Case 1).—Wall of the colon, demonstrating the inflammatory thickening of submucosal tissues, with numerous intralymphatic leucocyte masses, particularly at the border of submucosal and muscular layers. Hematoxylin and eosin; × 24.

Fig. 3 (Case 1).—Intralymphatic leucocyte nodule in the capsule of a mesenteric lymph node; × 150.

Fig. 4 (Case 2).—Ileocecal valve, near bottom of the illustration, and thickening of the wall of the opened cecum and ascending colon. The edematous thickening of adjacent omentum is shown. Reproduction of a Kodachrome transparency.

Fig. 5 (Case 2).—Superficial ulceration of the colon. The inflammation and fibrosis of the submucosa are demonstrated, as well as the intralymphatic leukocyte masses; × 12.

CASE 2 (A52-444).—A black cocker spaniel, male, one year old, was purchased from a pet shop. For three months the dog had an intermittent bloody diarrhea. Fecal examination was negative for parasites. The white blood cell count was 21,750 per cubic millimeter, with segmented polymorphonuclear leucocytes 55%, stab forms 23%, eosinophiles 12%, lymphocytes 9%, and monocytes 1%. The animal was killed.

At autopsy the terminal ileum was slightly thickened in the last 6 cm. The entire wall appeared involved, with narrowing of the lumen. The mucosa was reddened and swollen. The

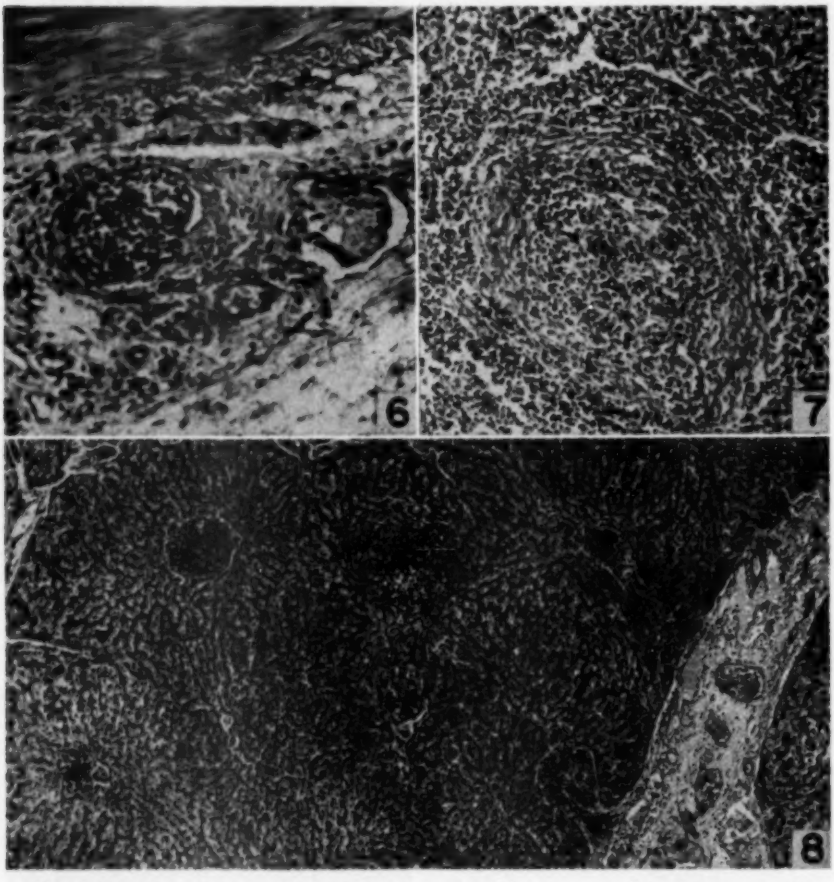


Fig. 6 (Case 2).—Subserosal lymphatics, irregularly dilated or containing leucocyte nodules, from the cecum; × 150.

Fig. 7 (Case 2).—Granulomatous nodule in a mesenteric lymph node; × 100.

Fig. 8 (Case 2).—Hepatic granulomas. The leucocytic nodules lie in a central vein, as shown at upper left, and other granulomas appear to involve lymphatics; × 50.

cecum and proximal 4 cm. of ascending colon were similarly but slightly more markedly thickened and with adhesions between the serosa and omentum (Fig. 4). The terminal descending colon and rectum were more markedly thickened up to 2 cm., with narrowing of the lumen. On section the feces were bloody and soft. The entire mucosal surface was mottled red and yellow with indistinct diffuse ulcerations. The wall was gray-white and firm, with slightly injected serosa. The regional lymph nodes all appeared enlarged and edematous.

The uninvolved intervening colon showed only slight dilatation. No other visceral abnormalities were observed grossly.

Microscopically leucocytic infiltration and thickening of the colonic submucosa were identical with Case 1. The process was severer and more advanced, with superficial mucosal ulcerations covered with fibrin in which bacteria were found (Fig. 5). Bacteria were also seen superficially in adjacent submucosa. Lymphatics were locally partly or completely plugged by masses of leucocytes and hyperplastic desquamated endothelial cells (Fig. 6). The edematous submucosa contained increased numbers of enlarged fibroblasts indicative of early cicatrization. There was hyperplasia of the normal colonic lymphoid aggregates, and bases of glands extended down farther than usual into these areas.

Regional lymph nodes were markedly hyperplastic, with acute inflammation, foci of necrosis, and proliferation of large macrophages in sinusoids. A few early granulomas, rarely with giant cells, were identified (Fig. 7). Macrophages locally contained vacuoles, probably lipids.

In the liver, at the edges of two portal zones, microscopic inflammatory foci were found projecting into hepatic lobules. They were composed of macrophages within and outside lymphatics, with eosinophiles and polymorphonuclear leucocytes (Fig. 8). There was associated focal hepatic necrosis.

Sections of heart, lung, spleen, pancreas, stomach, adrenal gland, kidney, and muscle showed no inflammatory lesions. Bone marrow appeared hyperplastic but without a relative excess of eosinophiles.

All sections of the two animals, stained by hematoxylin and eosin or Giemsa methods, were carefully searched for parasites, their larvae or ova, bacteria, fungi, or viral inclusion bodies. None were found except as noted above. In Case 2 there were found two aggregates of unidentified brown pigment and one crystalline foreign body in the ulcerations. No other foreign material was identified.

COMMENT

From the viewpoints of clinical signs, x-ray changes, gross and microscopic abnormalities, the two cases reported appear similar. They likewise closely simulate pathologically the human disease regional ileitis (terminal ileitis, cicatrizing enterocolitis, regional enteritis) and the disease condition of this type described in swine.

The morphologic sequences in the development of human regional enterocolitis have been fairly well established.† In early or acute cases there is a severe cellulitis, with infiltration particularly by macrophages and eosinophiles. Later in the more chronic cases granulomas are formed, with chronic inflammation and cicatrization of intestinal wall. The most important inflammatory localization in cicatrizing enterocolitis is in lymphatics, where exudation is followed by obliterative lymphangitis with production of what is essentially a localized intestinal elephantiasis. Exudative and granulomatous lesions of comparable types occur simultaneously in the adjacent mesentery and regional lymph nodes. Granulomas in the liver have occasionally also been observed.

In the dogs studied, all these processes were identified. The dogs were killed relatively early in the disease course, when the granulomas were beginning to be evident. As is also true in children, acute regional enterocolitis is more severe in

† References 9 and 10.

small animals, perhaps because of the smaller caliber of their intestinal lumens. Edema and exudation are thus more likely to produce severe early disabling symptoms.

The predominant localization beyond the ileocecal valve in the reported cases is less common in human patients but is observed. The two dogs demonstrated "skip areas" of involvement in the colon, which is a peculiarity of the condition in man. Emsbo reported that advanced terminal ileitis cases in swine also involved the cecum and colon and sometimes the jejunum.

The presence of blood eosinophilia in Case 2 and striking tissue eosinophilia in the two dogs would at first suggest either a parasitic infestation or an allergic reaction. However, careful search of stools and tissues failed to uncover any parasites, ova, or larvae in these animals. Eosinophilia may suggest hypersensitivity but is not a proof of its participation. In subacute human regional enteritis, eosinophilia of the intestinal wall is also observed but not to the degree found in the dogs.

Reichert and Mathes, in their close experimental simulation of regional enteritis, failed to elicit the same severity of leucocytic exudation as was observed in the naturally occurring cases reported. The spontaneous reaction of tissues was more diffuse, and secondary leucocytic collections to resist bacterial infection would logically be expected to be more outspoken in the naturally developing instances of regional enterocolitis.

No new suggestion as to etiology has arisen from observations of the canine or porcine cases. The presence microscopically of an evident abnormal response to lipid suggests both in dogs and men some derangement of intestinal lipid absorption and its further transport. It is a common observation that some cocker spaniels are excessively and at times painfully nervous dogs, which may be of interest to those who feel psychosomatic factors to be important in the development of regional enterocolitis.

SUMMARY

Two cases of regional enterocolitis have been reported in cocker spaniel dogs. Grossly and microscopically the disease appeared comparable to regional ileitis in humans, and to terminal ileitis as described in swine. The pathogenetic and etiologic factors are briefly considered.

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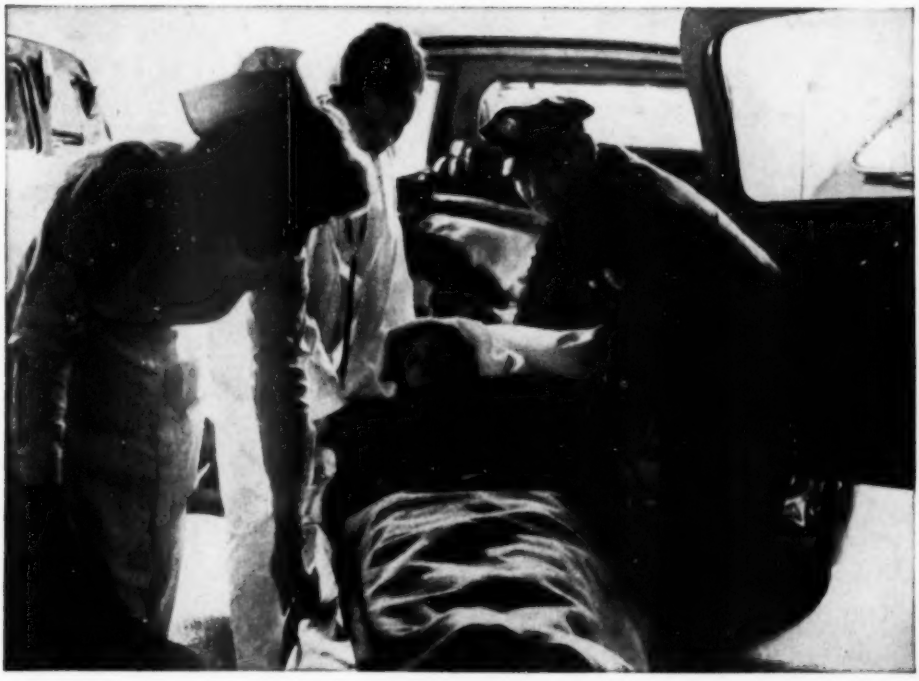

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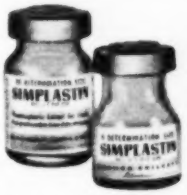

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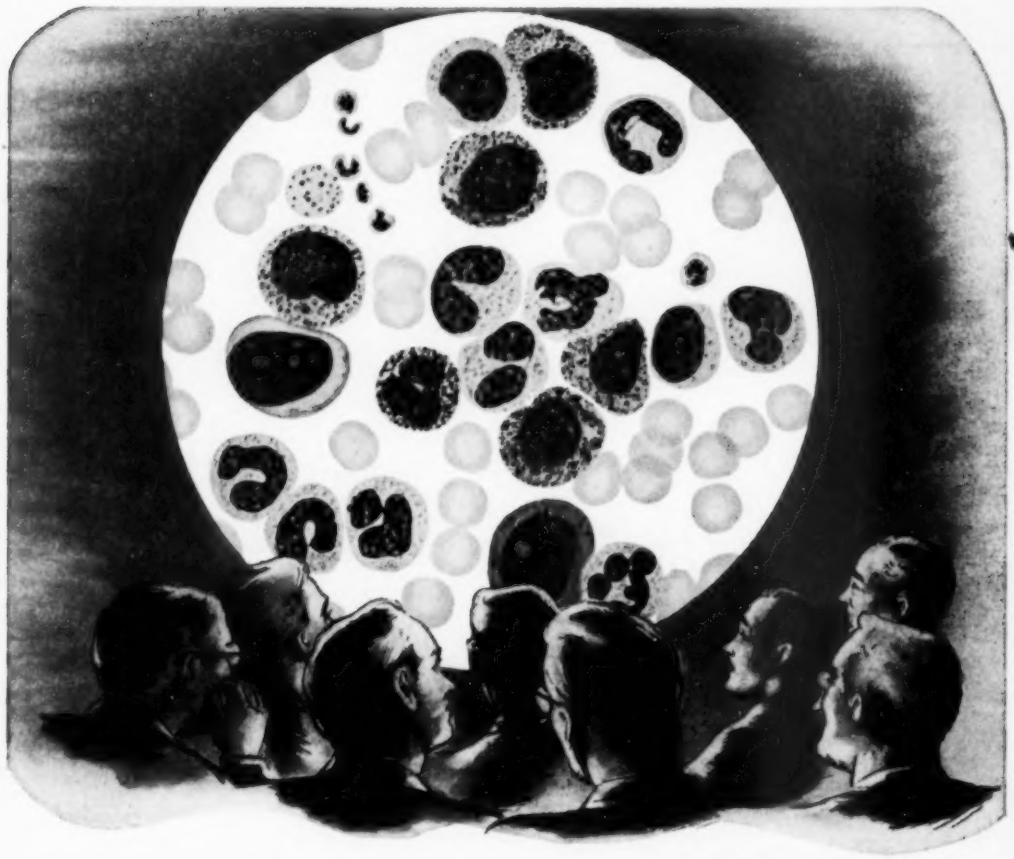

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